Reference Series in Biomedical Engineering
Tissue Engineering and Regeneration
Series Editor: Heinz Redl

SPRINGER REFERENCE

Jeffrey M. Gimble · Darja Marolt Presen
Richard O. C. Oreffo · Susanne Wolbank · Heinz Redl
Editors

Cell Engineering and Regeneration

termis®

Springer

Reference Series in Biomedical Engineering

Tissue Engineering and Regeneration

Series Editor

Heinz Redl
Ludwig Boltzmann Institute for Experimental and
Clinical Traumatology/AUVA Research Center
Austrian Cluster for Tissue Regeneration
Wien, Austria

This series Tissue Engineering and Regeneration consists of comprehensive reference texts encompassing the biological basis of tissue regeneration, basic principles of tissue engineering, and the current state-of-the-art in tissue engineering of specific tissues and organs. Each volume combines established fundamentals and the latest developments, thus forming an invaluable collection for both experienced researchers as well as practitioners from other areas of expertise. The spectrum of topics ranges from the use of cells for tissue regeneration and tissue engineering, growth factors and biological molecules affecting tissue development and regeneration, to the specific roles of biophysical factors in tissue development and regeneration.

Tissue engineering lies at the crossroads of medicine, life sciences, and engineering. The field has developed extensively over the last two decades, addressing the requirements of tissue and organ replacement as well as regeneration in a variety of congenital, traumatic, disease, and aging-related conditions, including some of the most critical unmet challenges in modern medicine. Both our increased understanding of the biological basis of tissue engineering as well as significant technological advances mean that engineering design principles can now be used for the de novo construction of functional tissue replacements that meet the requirements of research and clinical applications.

More information about this series at http://www.springer.com/series/13441

Jeffrey M. Gimble • Darja Marolt Presen
Richard O. C. Oreffo • Susanne Wolbank
Heinz Redl
Editors

Cell Engineering and Regeneration

With 86 Figures and 17 Tables

Springer

Editors
Jeffrey M. Gimble
Tulane Univ. Sch. of Medicine
New Orleans, LA, USA

Darja Marolt Presen
AUVA Trauma Research Center
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
Vienna, Austria

Austrian Cluster for Tissue Regeneration
Vienna, Austria

Richard O. C. Oreffo
Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences
University of Southampton
Southampton, UK

Susanne Wolbank
AUVA Trauma Research Center
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
Vienna, Austria

Austrian Cluster for Tissue Regeneration
Vienna, Austria

Heinz Redl
AUVA Trauma Research Center
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
Vienna, Austria

Austrian Cluster for Tissue Regeneration
Vienna, Austria

ISBN 978-3-319-08830-3 ISBN 978-3-319-08831-0 (eBook)
ISBN 978-3-319-08832-7 (print and electronic bundle)
https://doi.org/10.1007/978-3-319-08831-0

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This book is dedicated to all our colleagues in the TERM field and beyond involved in basic research or dedicated to clinical applications and last but not least to all the patients, young and old, who urgently need the support of regenerative medicine.

Preface

This Springer Reference updateable book series on Tissue Engineering and Regenerative Medicine describes the state of the art in the field of TERM and allows, with the electronic online platform adopted, continuous updates and editions to ensure the information is relevant, up to date, and can serve as reference for the field. The books can be used in research, teaching, and clinical translation in order to improve the quality of life and survival of our patients.

Within the field of tissue engineering, the combination of cells, biomaterials, and growth factors is required for tissue reparation and regeneration. Nevertheless, it is worth highlighting that cells, stem-progenitor, and differentiated progeny are the key elements in all tissue engineering and regenerative medicine (TERM) applications. It is the cell population that, ultimately, will facilitate the repair and regeneration of lost, damaged, or traumatized tissue. Therefore, we have dedicated a whole book to this central topic with chapters detailing stem, progeny, and differentiated cell populations, including function and application.

In recent years, emerging evidence has uncovered an important role for the cell "secretome" and the various cell factors released and generated in repair and cell signaling. It may prove that this mechanism may supplant the role of the transplanted cells themselves as the key factor in cell and tissue regeneration. In particular, the "new world" of extracellular vesicles and their cargo has opened up a new horizon of therapeutic agents that are potentially easier to manufacture, distribute, and apply.

We are, of course, aware of the limitations of any one book in trying to cover all potential cell sources; therefore, it was not possible to include some chapters (e.g., amniotic fluid cells, cryopreservation) in this first edition – these chapters will be available digitally, in due course, as per the innovative online concept of this book series.

Acknowledgments

We would like to thank all the authors and co-editors for their contribution, support, and suggestions. Special thanks to our reviewers (for the list of reviewers, see below). We would also like to thank the Tissue Engineering and Regenerative Medicine International Society (TERMIS) for their support of this tissue engineering series. Finally, our special thanks go to Ester Venturato and Susanne Windwarder, who have been instrumental in the success of this book series, as well as to the team at Springer for their vision, ongoing help, and professional work.

List of Reviewers

Christiane Fuchs, David Hercher, Colin Jahoda, Cornelia Kasper, Gerhard Krumschnabel, Antonina Lavrentieva, Christina Schuh, Tudorita Tumbar

Contents

About the Editors

Jeffrey M. Gimble is Co-founder and Chief Scientific Officer of LaCell LLC and Obatala Sciences Inc. For over 30 years, Dr. Gimble's laboratory has focused on stromal/stem cells isolated from bone marrow and adipose tissue as a model system for differentiation, hematopoiesis, metabolism, and regenerative medicine. Over this period, he has held faculty appointments (full time or adjunct) at the Oklahoma Medical Research Foundation, University of Oklahoma Health Sciences Center, Baylor College of Medicine, University of North Carolina Chapel Hill, Duke University Medical Center, Louisiana State University Health Sciences Center New Orleans, and Tulane University Center for Stem Cell Research and Regenerative Medicine. In addition, Dr. Gimble spent 4 years in the Research Triangle Park, NC, at Zen-Bio, Inc., a biotechnology company supplying human primary adipose-derived cells to the research community (1999–2000), and at Artecel Sciences, a tissue engineering company that he co-founded and helped lead as Chief Scientific Officer (2000–2003).

Darja Marolt Presen completed her doctoral studies at the University of Ljubljana, Slovenia, and was a visiting Ph.D. student at the Massachusetts Institute of Technology, Boston, USA. She trained as a postdoctoral scientist at Columbia University, New York, USA, and was leading the bone regeneration group at the New York Stem Cell Foundation Research Institute, New York, USA. She is currently the principal investigator of the bone bioengineering and regeneration group at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in Vienna, Austria. Her research in bone tissue engineering and regeneration was supported

by fellowships and grants from the Republic of Slovenia, New York Stem Cell Foundation, FFG Austria, and European Commission (Horizon 2020).

Richard O. C. Oreffo is Professor of Musculoskeletal Science and Co-founder/Director of the Centre for Human Development, Stem Cells and Regeneration at the University of Southampton. He leads a multidisciplinary research group focused on developing strategies to repair bone and cartilage, with translation a key personal driver. To achieve these goals, he has brought together and developed teams of clinicians/life scientists over the last 20 years and trained 47 M.D./Ph.D. students to completion, to date. He has published more than 300 peer-reviewed paper including breakthrough publications on skeletal stem cells and nanotopography, bone regeneration, as well as epigenetics and holds 6 patents. Richard is Founder, Director, and CSO of Renovos Biologics Limited, a Fellow of the Institute of Biology, and in 2015 was awarded a Doctor of Science degree by the University of Oxford and elected a Fellow of International Orthopaedic Research (FIOR) in 2019.

Susanne Wolbank is Head of the Stem Cell Biology Lab and, since 2019, Deputy Director at the Ludwig Boltzmann for experimental and clinical traumatology, Vienna, Austria. She is a biotechnologist (University of Natural Resources and Applied Life Sciences, Vienna) and held a postdoc position at the Clinical Research Center, Karolinska Institutet, Stockholm, on in vitro and in vivo characterization of human embryonic stem cells. She now combines her technological background with her basics knowledge on stem cells to investigate cellular aspects of tissue engineering and regenerative medicine. Her main research interest lies in stem cells from human "waste materials" such as perinatal tissues or adipose tissue. Her work has focused on optimizing these therapeutically relevant cells from isolation to improving the mode of application by activation via shockwave, cell-free application, combination with suitable biomaterials, and characterizing these cells including their interaction with other regenerative cell types.

She is the current General Secretary of the International Placenta Stem Cell Society (IPLASS).

Heinz Redl Ludwig Boltzmann Institute for Clinical and Experimental Traumatology.

Heinz Redl has a background in biochemistry with almost 40 years' experience in trauma and regenerative medicine research. He was Director of the Ludwig Boltzmann Institute of Experimental and Clinical Traumatology within the main trauma research center of AUVA (1998–2019) representing 7 trauma and 4 rehabilitation centers and holds the position of Associated Professor at the Technical University Vienna, Institute for Chemical Engineering, plus adjunct Professor at the University of Texas, Medical Branch at Galveston and at the Medical University of Vienna. He coordinates the Austrian Cluster for Tissue Regeneration since 2006, which includes 28 work groups from academia with multiple research targets and 12 spin-off groups. To further enhance industry cooperation, he founded the company Trauma Care Consult in 1998, which specializes in preclinical research and assists product registration at FDA and EMA. In 2014 he co-founded the spin-off company Liporegena and in 2017 MorphoMed. Prof. Redl organized many conferences in the field of regenerative medicine such as the World Congress for Tissue Engineering and Regenerative Medicine (TERMIS 2012) and many annual workshops (Winterschool Radstadt and Wiggers Bernhard series). He holds positions in several societies, such as Past-Chair of TERMIS-EU, and he was awarded "International Fellow of Tissue Engineering & Tissue Regeneration" in 2015. He is Editor in Chief of the updatable book series Springer/TERMIS "Tissue Engineering and Regeneration." His expertise includes experience in different fields of tissue regeneration, being a co-developer of the fibrin sealant system (>40 years), developing surgical devices in current clinical use, and several collaboration projects with major industry partners.

Contributors

Rita Aires CRTD Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany

Hendrik Jan Ankersmit Cristian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Vienna, Austria

Head FFG Project 852748 "APOSEC", Vienna, Austria

Department of Thoracic Surgery, Medical University Vienna, Vienna, Austria

Cedric Badowski Institute of Medical Biology, A*STAR, Singapore, Singapore

Asmita Banerjee Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center, Vienna, Austria

Andrea Barbero Department of Biomedicine, University of Basel and University Hospital of Basel, Basel, Switzerland

Lucian Beer Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna, Austria

Cristian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Vienna, Austria

Johanna Bolander Tissue Engineering Laboratory, Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium

Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

Paul E. Bourgine Cell, Tissue and Organ Engineering Laboratory, Department of Clinical Sciences Lund, Lund University, Lund, Sweden

Lund Stem Cell Centre, Lund University, Lund, Sweden

Wallenberg Centre for Molecular Medicine, Lund University, Lund, Sweden

Daniel R. Brison Faculty of Biology Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK

Mats Brittberg Cartilage Research Unit, University of Gothenburg, Region Halland Orthopaedics, Kungsbacka Hospital, Kungsbacka, Sweden

Susan J. Burke Basic Sciences/Immunogenetics, Pennington Biomedical Research Center, Baton Rouge, LA, USA

Verena Charwat Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

Chih-Chiang Chen Department of Dermatology, Taipei Veterans General Hospital, Taipei, Taiwan

Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan

Department of Dermatology, National Yang-Ming University, Taipei, Taiwan

Maria Chiara Ciuffreda Coronary Care Unit, Laboratory of Experimental Cardiology for Cell and Molecular Therapy, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

Andreas Clementi Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

J. Jason Collier Basic Sciences/Islet Biology, Pennington Biomedical Research Center, Baton Rouge, LA, USA

Pamina Contreras-Kallens Centro de Medicina Regenerativa, Facultad de Medicina, Clínica Alemana – Universidad del Desarrollo, Santiago, Chile

Diego Correa Department of Orthopaedics, Division of Sports Medicine, Diabetes Research Institute, Cell Transplant Center, University of Miami, Miller School of Medicine, Miami, FL, USA

Department of Biology, Skeletal Research Center, Case Western Reserve University, Cleveland, OH, USA

Caroline M. Curtin Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland

Advanced Materials and Bioengineering Research Centre (AMBER), RCSI and TCD, Dublin, Ireland

John E. Davies Institute of Biomaterials and Biomedical Engineering, Toronto, ON, Canada

Faculty of Dentistry, Toronto, ON, Canada

Cristian De Gregorio Centro de Medicina Regenerativa, Facultad de Medicina, Clínica Alemana – Universidad del Desarrollo, Santiago, Chile

Ram V. Devireddy Department of Mechanical Engineering, Louisiana State University, Baton Rouge, LA, USA

Mari Dezawa Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, Sendai, Japan

Solvig Diederichs Research Center for Experimental Orthopaedics, Heidelberg University Hospital, Heidelberg, Germany

Ruslan I. Dmitriev Metabolic Imaging Group, School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland

Dominik Egger Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

Tosan E. Ehanire Department of Surgery, Division of Plastic and Reconstructive Surgery, University of Florida, College of Medicine, Gainesville, FL, USA

Diana Gaspar Regenerative, Modular and Developmental Engineering Laboratory (REMODEL) and Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM), Biomedical Sciences Building, National University of Ireland Galway, Galway, Ireland

Renate Gehwolf Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

Massimiliano Gnecchi Coronary Care Unit, Laboratory of Experimental Cardiology for Cell and Molecular Therapy, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

Department of Molecular Medicine, Unit of Cardiology, University of Pavia, Pavia, Italy

Mary B. Goldring Hospital for Special Surgery and Weill Cornell Medical College, HSS Research Institute, New York, NY, USA

Johannes Grillari Department of Biotechnology, BOKU - University of Natural Resources and Life Sciences Vienna, Vienna, Austria

Moritz Grütz Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

Mariann Gyöngyösi Division of Cardiology, Medical University of Vienna, Vienna, Austria

Tim Hardingham Wellcome Centre for Cell-Matrix Research, Division of Cell-Matrix Biology and Regenerative Medicine, School of Biology, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK

John W. Haycock Department of Materials Science and Engineering, The University of Sheffield, Sheffield, UK

Tim Herpelinck Tissue Engineering Laboratory, Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium

Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

Jin-Bon Hong Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan

Wen-Yen Huang Department of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei, Taiwan

Aneesa Iskander Institute for Chemistry and Biotechnology (ICBT), Department Life Sciences and Facility Management, ZHAW Zurich University of Applied Sciences, Wädenswil, Switzerland

Florien Jenner University Equine Hospital, University of Veterinary Medicine Vienna, Vienna, Austria

Cornelia Kasper Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

Adam J. Katz Department of Surgery, Division of Plastic and Reconstructive Surgery, University of Florida, College of Medicine, Gainesville, FL, USA

Armand Keating Institute of Biomaterials and Biomedical Engineering, Toronto, ON, Canada

University of Toronto, Toronto, ON, Canada

Cell Therapy Program, University Health Network, Toronto, Canada

Arthritis Program, Krembil Research Institute, University Health Network, Toronto, Canada

Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada

Sean D. Keeley CRTD Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany

Elisabeth Kemter Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences; and Center for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Munich, Germany

Susan J. Kimber Faculty of Biology Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK

Alexander Kind Chair for Livestock Biotechnology, TUM School of Life Sciences Weihenstephan, Technische Universität München, Freising, Germany

Christine Lehner Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

Mingxing Lei 111 Project Laboratory of Biomechanics and Tissue Repair, College of Bioengineering, Chongqing University, Chongqing, China

Philip Lewis Faculty of Biology Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK

Sung-Jan Lin Department of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei, Taiwan

Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan

Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan

Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan

Frank P. Luyten Tissue Engineering Laboratory, Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium

Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

Darja Marolt Presen AUVA Trauma Research Center, Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

Irene Mencía Castaño Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland

Advanced Materials and Bioengineering Research Centre (AMBER), RCSI and TCD, Dublin, Ireland

Michael Mildner Department of Dermatology, Medical University of Vienna, Vienna, Austria

Michael G. Monaghan Department of Mechanical and Manufacturing Engineering and Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland

Advanced Materials for BioEngineering Research (AMBER) Centre, Trinity College Dublin and Royal College of Surgeons in Ireland, Dublin 2, Ireland

Manuela Mura Coronary Care Unit, Laboratory of Experimental Cardiology for Cell and Molecular Therapy, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

Department of Molecular Medicine, Unit of Cardiology, University of Pavia, Pavia, Italy

Roberto Narcisi Department of Orthopaedics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Nuno Neto Department of Mechanical and Manufacturing Engineering and Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland

Sylvia Nuernberger Department of Trauma Surgery, Medical University of Vienna, Vienna, Austria

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

Fergal J. O'Brien Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland

Advanced Materials and Bioengineering Research Centre (AMBER), RCSI and TCD, Dublin, Ireland

Richard O. C. Oreffo Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton, UK

Johanna Pruller Randall Centre for Cell and Molecular Biophysics, King's College London, London, UK

Rosanne M. Raftery Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland

Advanced Materials and Bioengineering Research Centre (AMBER), RCSI and TCD, Dublin, Ireland

Michael Raghunath Institute for Chemistry and Biotechnology (ICBT), Department Life Sciences and Facility Management, ZHAW Zurich University of Applied Sciences, Wädenswil, Switzerland

Mark A. Randolph Harvard Medical School, Massachusetts General Hospital, Boston, MA, USA

Heinz Redl AUVA Trauma Research Center, Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

Bruno Reichart Walter-Brendel-Center of Experimental Medicine, Ludwig-Maximilians-Universität München, Munich, Germany

Ana Maria Sandoval-Castellanos Department of Materials Science and Engineering, The University of Sheffield, Sheffield, UK

Tatiana Sandoval-Guzmán CRTD Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany

Center for Healthy Aging, Medical Faculty Carl Gustav Carus Technische Universität Dresden, Dresden, Germany

Christina M. A. P. Schuh Centro de Medicina Regenerativa, Facultad de Medicina, Clínica Alemana – Universidad del Desarrollo, Santiago, Chile

Edina Silajdžić Faculty of Biology Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK

Elisabeth Simader Cristian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Vienna, Austria

Department of Thoracic Surgery, Medical University Vienna, Vienna, Austria

Daniel Tang Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton, UK

Herbert Tempfer Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

University of Salzburg, Salzburg, Austria

Andreas Traweger Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

University of Salzburg, Salzburg, Austria

Gerjo J. V. M. van Osch Department of Orthopaedics and Department of Otorhinolaryngology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

John T. Walker Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, ON, Canada

Adelheid Weidinger Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center, Vienna, Austria

Eckhard Wolf Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences; and Center for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Munich, Germany

German Center for Diabetes Research (DZD), Neuherberg, Germany

Peter S. Zammit Randall Centre for Cell and Molecular Biophysics, King's College London, London, UK

Dimitrios I. Zeugolis Regenerative, Modular and Developmental Engineering Laboratory (REMODEL) and Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM), Biomedical Sciences Building, National University of Ireland Galway, Galway, Ireland

Janja Zupan Department of Clinical Biochemistry, Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

Part I

Cell Sources

Overview of Cell Types Capable of Contributing to Skeletal Muscle Repair and Regeneration

Johanna Pruller and Peter S. Zammit

Contents

Abstract

Skeletal muscle is the most abundant tissue in the human body, accounting for more than 30% of body weight, and is vital for the maintenance of posture, locomotion, and breathing. Skeletal muscle possesses a high intrinsic regenerative ability due to its resident stem cell, the satellite cell. However, muscle repair can fail in muscular dystrophies, during aging or after extensive trauma. A multitude of cell types are currently under investigation for their ability to support muscle regeneration in pathologies that induce tissue damage beyond the capacity of physiological regeneration. Even if the satellite cell is the most potent myogenic cell, factors including limited availability, difficulty of isolation, and in vitro expansion potential need to be addressed, and unorthodox cell types investigated, to develop the optimal cell type for a given therapeutic application. This chapter gives an overview of the advantages and disadvantages of cell types

J. Pruller (✉) · P. S. Zammit (✉)
Randall Centre for Cell and Molecular Biophysics, King's College London, London, UK
e-mail: johanna.prueller@kcl.ac.uk; peter.zammit@kcl.ac.uk

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_2

with myogenic capacity, focusing primarily on satellite cells, bone marrow-derived cells, muscle interstitial cells, and pluripotent stem cells.

1 Introduction

Skeletal muscle provides the body with the ability for controlled movement and can normally effectively repair and regenerate to counter various insults. However, failure to regenerate can occur in diseases including muscular dystrophies, trauma that severely damages a large area of a muscle (such as in road traffic accidents), or removal as a consequence of surgery, all of which are associated with disability. There is much investigation into how skeletal muscle can be repaired in response to this larger-scale damage, and the best cell source to use to repair skeletal muscle after such trauma. Here we review the properties of the resident skeletal muscle stem cell, the satellite cell (Katz 1961; Mauro 1961), and noncanonical cell types with myogenic potential. These unorthodox sources include CD133 cells (Negroni et al. 2009), bone marrow-derived stem cells (Gussoni et al. 2002), muscle interstitial cells (Cottle et al. 2017) such as pericytes (Birbrair et al. 2013a) and mesoangioblasts (Galvez et al. 2006), and myogenic differentiation of embryonic stem cells (Dinsmore et al. 1996) and induced pluripotent stem cells (Chal et al. 2016). Even if the satellite cell is the most potent myogenic cell (Relaix and Zammit 2012), factors such as low number, degree of difficulty of isolation, limited in vitro expansion, clinical safety, and sensitivity to genetic manipulation have to be considered against the properties of unorthodox myogenic sources when deciding the optimal cell type for a given therapeutic application (Meng et al. 2011). Here, we give an overview of the main cell types that can contribute to skeletal muscle regeneration in mouse and man, their isolation, and potential therapeutic application.

2 Skeletal Myogenesis

Skeletal muscle accounts for ~30% of the body mass of an adult woman and ~38% for men, with its primary functions being maintenance of posture, breathing, and locomotor activity (Janssen et al. 2000). It also has important roles in metabolism and thermoregulation. The basic contractile unit of skeletal muscle is the muscle fiber, containing myofibrils composed of sarcomeres arranged in series. Sarcomeres, in turn, contain the actin and myosin filaments that produce force by sliding past each other (Hanson and Huxley 1953). This highly ordered arrangement of sarcomeres gives skeletal muscle its characteristic striated appearance. Myosin heavy chain isoforms have varying ATPase activities that determine the speed of myofiber contraction, so forming “fast” or “slow” myofibers. Muscle fiber types also have different metabolic profiles to meet these varying requirements for ATP (Pette and Spamer 1986). Adult myofibers are adaptable, being capable of undergoing changes in fiber size (hypertrophy/atrophy) as well as fiber type (slow to fast and vice versa) (Greising et al. 2012).

Muscle fibers form by fusion of mononucleated muscle progenitor cells (myoblasts) during somitic and primary myogenesis in embryonic development. More myoblasts then fuse and donate their nuclei to grow myofibers during secondary myogeneisis in the foetal and perinatal periods, finally creating syncytia often containing several hundreds of myonuclei (Mintz and Baker 1967). However, some "progenitors" remain associated with myofibers to form a resident stem cell compartment and are termed satellite cells (Ciciliot and Schiaffino 2010). These stem cells become mitotically quiescent and reside in a niche on the surface of the myofiber, under the surrounding basal lamina.

Developmental and regenerative myogenesis is governed at the transcriptional level predominantly by members of the basic helix-loop-helix (bHLH) myogenic regulatory factor (MRF) family of MyoD, Myf5, myogenin, and Mrf4 (Weintraub 1993), as well as members of the myocyte enhancer factor (MEF2) family (Bharathy et al. 2013). The importance of these factors is shown by the ability of MRFs to activate the skeletal myogenic program after being introduced into various cell types derived from the three germ layers (Takano et al. 1998). MRFs dimerize with ubiquitously expressed bHLH E-proteins, and these heterodimers act as transcription factors by binding to the conserved DNA sequence CANNTG (E-box) that is present in most muscle-associated enhancer and promoter sequences (Moncaut et al. 2013; Zammit 2017).

Skeletal muscle displays a robust regenerative ability, especially if the connective tissue remains relatively intact. A finely tuned cascade of molecular events, recapitulating many deployed during developmental myogenesis, is initiated upon injury that ultimately yields a regenerated, vascularized, innervated, and contractile muscle (Chargé and Rudnicki 2004), with force/power re-established by 3 weeks in mouse (Rosenblatt 1992). However, trauma resulting in significant loss of muscle tissue cannot be successfully regenerated, resulting in compromised skeletal muscle function and strength.

Muscle bulk is also lost during normal aging in a process called sarcopenia (Delbono 2011), as a secondary process in cancer patients (cachexia) (Acharyya et al. 2005), or vascular injuries (Glass 2010), and in degenerative muscle diseases such as muscular dystrophies (Vilquin et al. 2011).

3 Skeletal Muscle Regeneration

Mechanical injuries to skeletal muscle lead to muscle fiber destruction by disruption of the basal lamina and plasma membrane. This allows calcium inflow, leading to degeneration induced by autodigestion followed by apoptosis (Ceafalan et al. 2014). Muscle regeneration following these events can be divided into three overlapping stages: destruction and inflammatory phase, repair phase, and remodeling phase (Ciciliot and Schiaffino 2010).

The destruction and inflammatory phase occurs between day 1 and day 3 after injury. Torn blood vessels allow inflammatory cells, such as polymorphonuclear leukocytes including neutrophils, to invade the necrotic injury site. Additionally,

connective scar tissue, mainly composed of fibrin and fibronectin derived from the blood of the initial hematoma, is formed immediately after the injury. Only a few hours after the onset of injury, the leukocytes are replaced by monocytes, which eventually differentiate into macrophages. Early invading M1 macrophages are present in high numbers at the injury site, peaking approximately 3–4 days after damage, with their numbers declining thereafter. M1 macrophages have two important roles, removal of necrotic tissue and secretion of pro-inflammatory cytokines, most notably tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) and 1β (IL-1β) (Chazaud et al. 2009; Saini et al. 2016). A phenotype switch in invading macrophages then causes a population of M2 macrophages to appear at the site of injury at 2–4 days post damage. M2 anti-inflammatory macrophages secrete cytokines that are necessary to terminate inflammation, for example, interleukin 10 (IL-10). Additionally, M2 macrophages secrete factors that stimulate proliferation of satellite cells, as well as their myogenic differentiation (Chazaud et al. 2009; Saini et al. 2016), working together with growth factors released from the ruptured extracellular matrix and other cell types such as fibro-adipogenic precursors (Cantini et al. 2002; Järvinen et al. 2005).

The repair phase relies on satellite cells that activate and then undergo multiple rounds of proliferation to generate a pool of myoblasts, which subsequently fuse together, or into surviving myofibers, and hence repair myofiber damage (Zammit et al. 2002). In the remodeling phase, newly formed myofibers mature, and scar tissue is reorganized and contracts (Fukushima et al. 2001).

However, issues may arise during the regeneration process that causes aberrant remodeling of the muscle, such as a failure to resolve scar tissue. Myotubes within the same basal lamina may not fuse to each other, which could lead to clusters of small fibers. Myotubes may only fuse at one end, creating branched fibers (Ontell et al. 1982). This can lead to segmental necrosis, with a subsequent additional regenerative process at the damaged extremity of the myotube that might be impaired by scar tissue (Shen et al. 2005). Since the two myotubes that would have fused together are effectively separated, new myotendinous junctions might be formed (Baker and Poindextor 1991). Additionally, nascent myofibers can sometimes form outside the basal lamina, due to satellite cell migration or the contribution of non-muscle stem cells, and remain embedded in the interstitial tissue (Schmalbruch 1976).

4 The Satellite Cell: The Resident Stem Cell of Skeletal Muscle

Satellite cells were first reported in 1961, when Alexander Mauro observed an apparently quiescent cell lying on the surface of a myofiber but below the basal lamina, in frog and rat muscle (Mauro 1961). In the same year, similarly located cells were also described on intrafusal muscle fibers (Katz 1961). This specific location, on the edge of a myofibre, gave rise to the name satellite cell.

Satellite cells equate to approximately 2–5% of the number of myonuclei in mature muscle. There is a difference in amount of satellite cells per myofiber or

cross-sectional area between muscle fiber types though; in mouse, fast twitch muscle fibers (mainly IIb and IIx) of the extensor digitorum longus (EDL) have fewer associated satellite cells than slower twitch muscle fibers (IIa and I) of the soleus (Zammit et al. 2002). There is often a concentration of satellite cells at the neuromuscular junction in slow fibers in mouse and, at least in chicken, more satellite cells at the ends of the myofiber (Allouh et al. 2008). Number and potency of satellite cells can additionally be affected by exercise and age (Shefer et al. 2006, 2010).

Satellite cells are the primary source of myonuclei during postnatal development and early muscle growth, before becoming mitotically quiescent (Schultz et al. 1978). Their role then changes during adult life, when they are only sporadically required for muscle fiber homeostasis, fiber hypertrophy and repair (Zammit 2008). While the number of satellite cells drops significantly from postnatal to adult life, the adult population remains constant, even after multiple cycles of regeneration (Ceafalan et al. 2014). Indeed, the satellite cell pool can respond to 50 weekly cycles of repeated muscle damage with efficient regeneration (Luz et al. 2002). Why regeneration fails in muscle wasting diseases such as muscular dystrophy is unclear but is probably related to a deteriorating microenvironment (Aguennouz et al. 2011; Blau et al. 1983).

As stem cells, satellite cells maintain the regenerative pool via self-renewal (Collins et al. 2005). After injury, satellite cells are stimulated to activate and proliferate to generate large numbers of new myofibers within a few days (Whalen et al. 1990). Activation is governed by a cocktail of signals such as growth factors including HGF, FGF, and IGF and chemical signals including NO, derived from infiltrating cells and damaged myofibers. In mice, satellite cells ex vivo give rise to many $Pax7^{+}MyoD^{+}$ cells that are committed to myogenic differentiation, Pax7 expression then declines, and myogenin expression is induced as the cells irreversibly withdraw from the cell cycle and begin to differentiate (Zammit et al. 2004). Soon after exit from the cell cycle, myoblasts start to express structural proteins such as sarcomeric myosin and fuse into myotubes (Shefer et al. 2006). A small number of satellite cell progeny become $Pax7^{+}MyoD^{-}$ and return to quiescence to maintain the satellite cell pool (Zammit et al. 2004). Alternatively, it is proposed that the satellite cell pool is heterogeneous, with a small subset of self-renewing satellite "stem" cells responsible for generating satellite cells with myogenic commitment (Kuang et al. 2007; Tierney and Sacco 2016).

Identification of satellite cells based solely on endogenous molecular markers is complex (Table 1). The best and most commonly used marker for quiescent and activated satellite cells is the paired homeobox transcription factor Pax7 (Seale et al. 2000; Hernández-Hernández et al. 2017; Yin et al. 2013; Marg et al. 2014), since other markers used to characterize satellite cells, such as CD34 (Beauchamp et al. 2000) and syndecan-3/syndecan-4 (Cornelison et al. 2001; Pisconti et al. 2012; Sinha-Hikim et al. 2004), are additionally expressed by other cell types in skeletal muscle (Tedesco et al. 2010) (Table 1). Characterization of satellite cells on tissue sections can be performed using co-immunolabeling of Pax7 and laminin, to confirm their location under the basal lamina (Ortuste Quiroga et al. 2016). Not all markers recognize all satellite cells though, for example, $Myf5^{YFP}$ is expressed in a small

Table 1 Overview of markers used to identify and/or isolate murine and human satellite cells

Marker	Proportion of satellite cells (SC)	Cellular Localization	Human	Murine	Other notable/ relevant expression (mice)	Bulk isolation methods	Reference
Pax7	100% of quiescent and activated SCs	Nucleus	√	√	Central Nervous System	Cell sorting from GMO mice, e.g., mouse: $Pax7^{GFP}$	Seale et al. 2000; Zammit et al. 2006; Boldrin and Morgan 2012; Reimann et al. 2004; Sambasivan et al. 2009
Pax3	Quiescent SCs (only subset in certain muscles)	Nucleus	?	√	Melanocyte stem cells, brachial and femural arteries	Cell sorting from GMO mice, e.g., mouse: $Pax3^{GFP}$	Relaix et al. 2006; Goupille et al. 2011
Myf5	Most quiescent and proliferating SCs and myoblasts	Nucleus	√	√	Central Nervous System	Mouse: $Myf5^{nLacZ}$ cell sorting from GMO mice, e.g., $Myf5^{YFP}$	Tajbakhsh et al. 1996; Beauchamp et al. 2000; Bareja et al. 2014; Daubas et al. 2000
Syndecan-3 and -4	98% of quiescent and activated SCs	Plasma membrane	√	√	Brain, dermis, bone, smooth muscle, tumors	Cell sorting	Cornelison et al. 2001; Sinha-Hikim et al. 2004; Pisconti et al. 2012
VCAM-1	Quiescent and activated SCs	Plasma membrane	?	√	Activated endothelial cells	Cell sorting as part of panel of markers	Rosen et al. 1992
c-met	Quiescent and activated SCs	Plasma membrane	√	√	Many tissues and tumors	Not used	Cornelison and Wold 1997; Marg et al. 2014
Foxk1	Quiescent and activated SCs	Nucleus	?	√	Neurons	Not used	Garry et al. 2000
Cd34	Quiescent and activated SCs	Plasma membrane	√	√	Hematopoietic, endothelial, mast, and dendritic cells	Cell sorting as part of panel of markers	Beauchamp et al. 2000; Boldrin and Morgan 2012; Pisani et al. 2010

M-cadherin	Quiescent and activated SCs, myoblasts	Plasma membrane	√	√	N/A	Not used	Irintchev et al. 1994; Boldrin and Morgan 2012
Caveolin-1	Quiescent and activated SCs, myoblasts	Plasma membrane	√	√	Endothelial fibrous and adipose tissue	Not used	Gnocchi et al. 2009; Boldrin and Morgan 2012; Volonte et al. 2005
α7 integrin	Quiescent and activated SCs, myoblasts	Plasma membrane	√	√	Vessel-associated cells	Cell sorting as part of panel of markers	Blanco-Bose et al. 2001; Pawlikowski et al. 2009
β1 integrin	Quiescent and activated SCs	Plasma membrane	√	√	Many tissues	Cell sorting as part of panel of markers	Kuang et al. 2007; Xu et al. 2015
CD56 (NCAM)	Quiescent and activated SCs	Plasma membrane	√	√	Glia, neurons, natural killer cells	Cell sorting and beads	Betsholtz 2004; Agley et al. 2015
Cxcr4	Subset of quiescent SCs	Plasma membrane	√	√	HSCs, vascular endothelial cells, neuronal cells	Cell sorting as part of panel of markers	Sherwood et al. 2004; Bareja et al. 2014
Nestin transgene	Roughly 98% of quiescent SCs and myoblasts	Cytoplasmic GFP	N/A	√	Central and peripheral nervous system	Cell sorting from GMO mice	Day et al. 2007
SM/C2.6 antigen	Subset of quiescent SCs	Plasma membrane	X	√	Unknown	Cell sorting	Fukada et al. 2004, 2013
Calcitonin receptor	Adult satellite cells	Plasma membrane	?	√	Many tissues	Not used	Yamaguchi et al. 2012
Teneurin4	Quiescent SCs	Plasma membrane	?	√	Many tissues	Not used	Fukada et al. 2007

subset (Kuang et al. 2007), while *Pax3*^{eGFP} is expressed in a subpopulation in only certain muscles (Calhabeu et al. 2013; Relaix et al. 2006) (Table 1). It remains unclear though, whether such heterogeneity is related to the presence of dedicated satellite "stem" cells or reflects other factors such as altered stem cell characteristics e.g., through some cells having been activated fewer times or having undergone fewer divisions (Zammit 2017). Recent studies on gene expression in quiescent satellite cells have identified more potential markers that may help to resolve this issue (Machado et al. 2017; van Velthoven et al. 2017).

Isolation of satellite cells was first performed either from whole muscle extracts or single-cell suspensions (Kakulas et al. 1968; Pogogeff and Murray 1946; reviewed in Scharner and Zammit 2011). These explant cultures also contained non-myogenic cells including adipocytes, lymphocytes and fibroblasts. Pre-plating of dissociated cells on a non-collagen-coated dish allows enrichment of myogenic cells as fibroblasts preferentially adhere to this surface (Blau and Webster 1981). Another option is the fractionation of a cell suspension on Percoll density gradients (Richler and Yaffe 1970). A means to ensure purity of the population is to isolate satellite cells by explant culture from single myofibers, rather than muscle tissue. In this approach, a whole muscle, such as the EDL in mouse, is enzymatically digested to isolate individual myofibers with their associated satellite cells. Myofibers can then be plated, and large quantities of satellite cell-derived myoblasts soon surround the myofiber. This methodology can be successfully applied to both murine (Moyle and Zammit 2014) and human skeletal muscle (Marg et al. 2014).

Apart from explant cultures, techniques such as FACS and MACS can be used. Initially, FACS was performed to enrich for myogenic cells on the basis of cell shape (Baroffio et al. 1993) and also size/granularity (Montarras et al. 2005), with the surface marker neural cell adhesion molecule (NCAM) used in early studies (Walsh and Ritter 1981). Subsequently, FACS or MACS sorting of combinations of $CD45^{-}Sca\text{-}1^{-}CXCR4^{+}\beta1\text{-}integrin^{+}CD34^{+}c\text{-}met^{+}$ (Sherwood et al. 2004), $CD34^{+}CD45^{-}Sca\text{-}1^{-}$ (Montarras et al. 2005), syndecan-3^{+} and syndecan-4^{+} (Tanaka et al. 2009) from murine biopsies, or CD56+/Desmin+ for human biopsies (Agley et al. 2015) isolate a cell population highly enriched for satellite cells. Samples for sorting-based isolation techniques are commonly derived from adult muscle that is first cleared of non-muscle tissue (blood vessel, connective tissue, nerve bundles, and adipogenic tissue), mechanically minced and enzymatically digested. A collagenase-dispase enzyme mixture is normally used because dispase preserves surface antigens more effectively than trypsin or pronase (Yablonka-Reuveni 2011). A detailed protocol can be found in Motohashi et al. 2014.

Where available, an alternative to endogenous surface antigens for FACS is using mice genetically modified to express fluorescent proteins under control of various genes/promoters including *Pax3* (Montarras et al. 2005), *Pax7* (Bosnakovski et al. 2008), *Myf5* (Biressi et al. 2007), or *Nestin* (Day et al. 2007) (Table 1). Satellite cells from Pax3-GFP or Pax7-GFP mice are isolated according to their $GFP^{+}/CD34^{+}/CD45^{-}/Sca1^{-}$ profile; the presence of GFP is essential as it allows differentiation between endothelial ($CD34^{+}/GFP^{-}$) and myogenic cells (Montarras et al. 2005).

Current advances in cell profiling have allowed new markers for muscle satellite/stem cells to be identified, such as CD9, CD104 (integrin subunit beta 4) (Porpiglia et al. 2017), Asb5, and CD82 (Giordani et al. 2019) that can aid in the isolation of pure populations, but they are not yet in routine use.

The main advantage of direct isolation methods without extended culture steps was that the isolated satellite cells better maintained their regenerative ability, that was otherwise lost during explant culture: freshly isolated satellite cells show a significantly higher potential for regeneration than after in vitro expansion (Collins et al. 2005; Gilbert et al. 2010; Montarras et al. 2005). The high potential of "fresh" satellite cells in muscle regeneration was demonstrated in an elegant experiment showing that transplantation of a single myofiber with approximately seven associated satellite cells successfully regenerated and repopulated areas of skeletal muscle in dystrophic *mdx* mice (Collins et al. 2005). Transplantation of human fibers with their associated satellite cells into irradiated mouse muscle also results in robust engraftment, muscle regeneration, and proper homing of human $PAX7^+$ satellite cells to the stem cell niche (Marg et al. 2014). Short culture periods without subculture allows satellite cells to maintain their myogenic potency after transplantation (Ikemoto et al. 2007) but expanded cells perform poorly after transplantation compared to freshly isolated cells (Montarras et al. 2005). The loss of regenerative potential could be attributed to a reduction of "stem cell" characteristics and/or fraction during in vitro expansion, and gene expression changes drastically even during the isolation process (e.g., Machado et al. 2017). Such compromised potency is particularly problematic in therapeutic approaches, as it is necessary to expand the cells to obtain large amounts and myoblasts have not been effective in clinical trials for muscular dystrophies (reviewed in Negroni et al. (2011)). Similar to mice, only 5% of total myofiber nuclei in humans belong to satellite cells, and biopsies tend to be small, therefore minimizing the amount of useful satellite cells that can be obtained for therapeutic use (Lindström and Thornell 2009). The demonstration that viable and functional satellite cells can be obtained postmortem in man, however, opens up a potential new source (Latil et al. 2012).

Methods to increase the number/effectiveness of satellite cells for grafting without compromising their myogenic potential are thus important and include culture on soft hydrogels with a stiffness mimicking skeletal muscle (approximately 12 kPa) (Gilbert et al. 2010), artificial recreation of the native niche (Quarta et al. 2016), or manipulation of various signaling pathways such as prostaglandins (Ho et al. 2017) and p38 (Cosgrove et al. 2014).

Overcoming reduced potency due to in vitro expansion should facilitate widespread testing of satellite cells in clinical trials for degenerative diseases such as muscular dystrophies – an area that is currently lacking progress (Briggs and Morgan 2013). Even though transplantation of satellite cells has led to encouraging results in dystrophic mice, translation of these results into humans remains challenging (Negroni et al. 2016). Systemic delivery of autologous satellite cells or satellite cell-derived myoblasts is not currently possible, but it is an issue being addressed (e.g. Gerli et al. 2019). However, local intramuscular injection of in vitro expanded satellite cells might be effective in certain conditions. In patients suffering from

oculopharyngeal muscular dystrophy (OPMD), a major issue is difficulties swallowing due to wasting of the cricopharyngeal muscles. Intramuscular grafting of homologous expanded satellite cells into cricopharyngeal muscles during cricopharyngeal myotomy appears to increase quality of life and muscle function, although the relative contribution of surgery and grafting is currently unclear (Périé et al. 2014; Negroni et al. 2016).

5 CD133+ Cells

These are a subset of human cells characterized by expression of CD133, a transmembrane protein that is expressed at high levels in a variety of stem cells (Kobari et al. 2001; Zuba-Surma et al. 2009). Initially, contribution of $CD133^+$ cells to myogenesis was reported in $CD133^+$ circulating hematopoietic stem cells that were isolated from human blood samples (Torrente et al. 2004). Cultivated on a feeder layer of myogenic cells, $CD133^+$ cells express early myogenic markers such as Myf5, Pax7, and M-cadherin and fuse with the feeder cells to form mosaic myotubes. Later, $CD133^+$ cells were found in the interstitium of skeletal muscle, in addition to a location associated with satellite cells in neonatal healthy, as well as in postnatal, dystrophic muscle (Meng et al. 2014). $CD133^+$ cells are a heterogeneous population of multipotent stem cells, giving rise to several mesenchymal lineages in culture, and can contribute to muscle regeneration after grafting, but not after intravenous delivery (Meng et al. 2014). Grafted human skeletal muscle-derived $CD133^+$ cells disperse better in host muscle, with enhanced regenerative ability, compared to human myoblasts alone (Negroni et al. 2009). For isolation of tissue-derived $CD133^+$ cells, muscle biopsies can be enzymatically digested and red blood cells/dead cells removed from the single-cell suspension, before incubation with CD133 microbeads and MACS sorting (Meng et al. 2014).

$CD133^+$ cells do, however, have several drawbacks, including being rare, ~1% of mononucleated skeletal muscle cells (Benchaouir et al. 2007), being too fragile for FACS sorting (Meng et al. 2014), requiring a complex culture medium that is no longer commercially available (Negroni et al. 2009), as well as losing their myogenic potential after prolonged in vitro culture (Meng et al. 2014). Furthermore, MACS-based isolation of $CD133^+$ cells from cord blood is affected by significant variability in purity of the isolated population (10–85%) (Pelagiadis et al. 2012). Muscle-derived $CD133^+$ cells were used in a clinical trial, where autologous $CD133^+$ cells were grafted intramuscularly into the abductor digiti minimi of eight Duchenne muscular dystrophy (DMD) patients, resulting in an increase in capillaries per muscle fiber and a change in the ratio of slow to fast muscle fiber types (Torrente et al. 2007). This is despite the observation that DMD patient-derived $CD133^+$ cells exhibit a markedly decreased myogenic potential compared to those from a healthy individual (Meng et al. 2018).

6 Bone Marrow-Derived Stem Cells with Myogenic Potential

In 1998, Ferrari et al. reported that stem cells not resident in skeletal muscle could be involved in muscle regeneration. They demonstrated that bone marrow-derived stem cells (BMDCs) were capable of contributing nuclei to myofibers if injected into regenerating muscle of immunodeficient *scid/bg* mice (Ferrari et al. 1998). However, BMDCs could only contribute to muscle regeneration with satellite cells present. A few years later, LaBarge and Blau used a model causing irradiation-induced muscle damage, so ablating the endogenous satellite cell pool. Mice were then injected with an unfractionated bone marrow cell suspension expressing a fluorescent marker, into the tail vein. This study showed that BMDCs not only contributed to muscle regeneration; they additionally reoccupied the empty satellite cell niche and were capable of self-renewal, thereby becoming heritably myogenic (LaBarge and Blau 2002). However, depending on the intensity, irradiation-mediated ablation does not remove all satellite cells from their niche (Heslop et al. 2000), making it possible that regeneration attributed to BMDCs would not be as efficient if ablation had been absolute.

Contribution of BMDCs to total myonuclei is also vastly different between different muscles following bone marrow transplantation. For example, muscles including the intercostal muscles and tongue contain about 0.002% of BMDCs, while the frequency was 0.26% in the EDL, 0.06% in the tibialis anterior, and 5.2% in the panniculus carnosus. Data was collected 16 months post bone marrow transplant, showing that incorporation of circulating stem cells seems to occur naturally at different levels. However, injury might still be a factor in the different rates of incorporation, as the panniculus carnosus may be damaged by "scruffing" the mouse during handling so incorporates higher amounts of BMDCs than muscles subjected to less damage, such as tibialis anterior (Brazelton et al. 2003), even in the absence of acute injury (Naldaiz-Gastesi et al. 2016).

There is also evidence that BMDCs may contribute to skeletal muscle in man. Female patients that had received a bone marrow transplant from male donors 6–12 years previously, had rare skeletal muscle fibers (0.6%) with a nucleus that contained a Y chromosome. Although no Y chromosomes were present in the satellite cell niche on sections or in desmin-expressing myoblast cultures grown from isolated satellite cells, a rare Y chromosome-containing centrally located nucleus was detected, indicating that BMDCs had continued to add myonuclei to muscle (Strömberg et al. 2013). Thus, while BMDCs can contribute to muscle, it is extremely inefficient when the satellite cell pool is intact. In another study, a DMD patient was treated with a bone marrow transplant for X-linked severe combined immunodeficiency, and donor nuclei could still be found in 0.5–0.9% of myofibers 13 years after BMDCs transplantation (Gussoni et al. 2002). With the obvious caveat of a single patient, the efficiency of BMDCs incorporation into skeletal muscle appears unaffected by the stimulus of chronic muscle regeneration likely in the DMD patient.

The specific mechanism of how BMDCs contribute to muscle regeneration is not well understood, and a large proportion of BMDCs that incorporate into myofibers do not actually activate the myogenic program (Lapidos et al. 2004; Wernig et al. 2005). The translational consequence of BMDC transplant is seen when patients suffering from partial denervation in the elbow were treated with BMDC injection and tendon transfer. Subsequently, muscle fibrosis decreased by 52%, while myofiber diameter, the presence of satellite cells, muscle density, and motor unit amplitude all increased (Hogendoorn et al. 2014). The effect of BMDCs may be indirect, as intramuscular injection 7 days after injury lead to alteration of the immune response, by downregulation of IL-1b, IL-6, TNF-α, and TGF-β1 and upregulation of IL-10, resulting in overall better regeneration and revascularization (Helal et al. 2016). BMDCs can also generate an environment favoring muscle regeneration in minipigs – by stimulating local VEGF production and shifting the macrophage phenotype to one promoting muscle regeneration (Linard et al. 2018). Finally, BMDCs can give rise to $CD45^-/Sca\text{-}1^+/desmin^+$ cells capable of myogenic differentiation (Luth et al. 2008). These cells localize to both within and outside of the basal lamina of muscle fibers and can contribute to skeletal muscle regeneration (Kowalski et al. 2018). Due to their localization and shared marker profile, they are thought to be the origin of some interstitial muscle-derived stem cells (Dreyfus et al. 2004).

7 Muscle Interstitial Cells in Muscle Regeneration

The term muscle interstitial cells (MICs) encompasses a diverse set of cell types found in the interstitium of skeletal muscle, with the relationship of these various cell types to each other, still relatively unclear. Their location presumably allows MICs to sense mechanical or chemical changes to the skeletal myofibers. Most MICs are of mesoderm origin, often from the vasculature, and are generally assumed to interact with satellite cells, muscle fibers, immune cells, and fibroblasts in the event of muscle damage. MICs can be broadly divided into two main groups: cells that support muscle regeneration and those that may directly contribute myonuclei (Table 2).

Fibro-adipogenic precursors (FAPs) are a population of MICs that are defined by expression of specific marker combinations, which also dictate their isolation via FACS and so characterization. Two different groups defined cells belonging to this population as either $CD45^-/CD31^-/lin^-/Sca1^+/CD34^+$ or $CD45^-/CD31^-/lin^-/Sca1^+/\alpha7int^-$ (Joe et al. 2010), or $CD31^-/CD45^-/SM/C\text{-}2.6^-/PDGFR\alpha^+/PDGFR\beta^+$ mesenchymal progenitor cells (Uezumi et al. 2010). In general, FAPs are capable of differentiating into adipocytes, osteoblasts (with BMP7), and smooth muscle cells (using TGF-β) (Uezumi et al. 2011); they do not, however, differentiate into skeletal muscle cells if cultured in low serum conditions, or if transplanted into a skeletal muscle regenerative environment (Uezumi et al. 2010). Additionally, $CD45^-/CD31^-/lin^-/Sca1^+/CD34^+$ FAPs normally also express Osr1 at low levels, but Osr1 expression increases upon injury. This population contributes specifically to

Table 2 Muscle interstitial cells: characterization and contribution to myogenesis

Muscle interstitial cell types	Characterization	Direct contribution to myogenesis	Reference
FAP	$CD45^-/CD31^-/lin^-/Sca1^+/CD34^+$ (low Osr1)	No	Joe et al. 2010
	$CD45^-/CD31^-/lin^-/Sca1^+/\alpha7int^-$	No	Joe et al. 2010
	$CD31^-/CD45^-/SM/C\text{-}2.6^-/PDGFR\ \alpha^+/PDGFR\beta^+$	No	Uezumi et al. 2010
Side population	$Sca1^+\ CD4^-$, $CD5^-$, $CD8^-$, $CD11^-$, $CD45^-$, Gr-1^-, c-KIT^-	Yes	Gussoni et al. 1999
SP subpopulation/ main population	$CD34^+/Sca1^+$	Yes	Tamaki et al. 2002, 2003
Myoendothelial cells	$CD56^+/CD34^+/CD144^+$	Yes	Zheng et al. 2007
PICs (FAP subpopulation?)	$CD34^+/Sca1^+/Pax7^-/PDGFR\ \alpha^-$	Yes	Besson et al. 2011; Pannerec et al. 2013
PICs (FAP subpopulation?)	$CD34^+/Sca1^+/Pax7^-/PDGFR\ \alpha^+$	No	
$Twist2^+Pax7^-$	$Twist2^+Pax7^-$	Maintenance/ regeneration of fast IIb muscle fibers only	Liu et al. 2017

adipogenic infiltration and post injury resident FAPs (Stumm et al. 2018). During muscle regeneration, it is thought that FAPs indirectly interact with satellite cells, as proliferation of FAPs rapidly increases prior to satellite cell proliferation (Joe et al. 2010). Indeed, pharmacological inhibition of in vivo FAP expansion by the tyrosine kinase inhibitor Nilotinib results in a failure of satellite cells to expand in response to injury (Fiore et al. 2016). FAPs aid muscle regeneration by releasing diffusible factors or by remodeling the microenvironment using disintegrin and metalloprotease ADAM12 upon injury. However, production of extracellular matrix components such as collagen for fibrosis will limit muscle regeneration, so FAPs may also contribute to the debilitating phenotype of muscle-degenerating diseases such as muscular dystrophies (Uezumi et al. 2011). Consistent with this notion, ADAM12 suppresses muscle regeneration and increases fibrosis if overexpressed (Jørgensen et al. 2007).

MICs with inducible myogenic potential include several cell types. Side population (SP) cells were first identified as BMDS multipotent hematopoietic stem cells that were characterized by their ability to efflux the vital fluorescent DNA dye Hoechst 33342 (Goodell et al. 1996). While isolating cells from the muscle of wild-type mice, Gussoni et al. also found a subpopulation of cells able to efflux Hoechst 33342 (Gussoni et al. 1999). SP cells are characterized by being $Sca1^+$, but negative for bone marrow and lineage markers (CD4, CD5, CD8, CD11, CD45, Gr-1, c-KIT), and display a high hematopoietic potential (Gussoni et al. 1999;

Jackson et al. 1999). The conditions used for FACS isolation of SP cells, such as Hoechst dye concentration however, can influence the composition of the SP fraction, making them a heterogeneous population (Montanaro et al. 2004; Rivier 2004). The regenerative potential of SP cells was highlighted after transplantation into lethally irradiated *mdx* mice, where they gave rise to dystrophin-positive myofibers (Gussoni et al. 1999). SP cells are juxtaposed to the platelet endothelial cell adhesion molecule (PECAM) expressing endothelium and blood vessels. Additionally, SP do not express Pax7 or desmin and only undergo myogenic differentiation if either co-cultured with primary myoblasts or injected into regenerating muscle (Asakura et al. 2002).

A potential subpopulation of the SP cells was proposed by Tamaki et al. who described $CD34^+/Sca1^+$ cells with multi-lineage potential from the skeletal muscle interstitium that were negative for hematopoietic markers. These cells express bHLH factors myogenin and MyoD and contribute to postnatal de novo myofiber formation, as well as adipocytes and endothelial cells (Tamaki et al. 2002). However, it was later determined that SP cells made only an extremely minor contribution to myogenic and endothelial generation in vitro, with 99.9% coming from the main population (Tamaki et al. 2003).

Myoendothelial cells have also been isolated from man and express both satellite and endothelial cell markers (CD56, CD34, and CD144), being located between myofibers, but outside of the basal lamina. Myoendothelial cells efficiently regenerate skeletal muscle after transplantation and differentiate into myogenic, osteogenic, and chondrogenic cells in vitro (Zheng et al. 2007).

The stress mediator PW1 has been used to define a population of MICs with myogenic potential called PW1(+)/Pax7(−) interstitial cells (PICs) (Mitchell et al. 2010). PICs are isolated from minced muscle tissue by FACS, by gating first on $CD45^-$ cells, followed by selection of $CD34^+$ and $SCA1^+$ cells. Of that population, PW1 and either PDGFα-positive or PDGFα-negative PICs can be selected, with both populations lacking Pax7 expression (Besson et al. 2011; Pannerec et al. 2013). PICs self-renew and differentiate into myofibroblasts, muscle fibers, and can occupy the satellite cell niche (Mitchell et al. 2010) but can also differentiate into adipocytes and compromise muscle regeneration (Yao et al. 2016). PICs contributing to adipogenesis share expression of PDGFRα with FAPs, while PICs capable of contributing to muscle regeneration are negative for PDGFRα (Pannerec et al. 2013). It is now thought that some PICs are a subpopulation of FAPs, sharing lineage markers CD34 and Sca1. The myogenic potential of PICs relies on the eventual expression of Pax7, acquired shortly before differentiating, as PICs from $Pax7^{-/-}$ mice exclusively adopt a smooth muscle fate (Mitchell et al. 2010).

Finally, the MIC $Twist2^+Pax7^-$ cells contribute exclusively to the regeneration of adult-type IIb/x myofibers, but are not involved in primary or secondary myogenesis. Ablation of $Twist2^+Pax7^-$ cells in mouse leads to type IIb-specific fiber atrophy, indicating their importance in maintenance and regeneration of these subtypes of fast muscle fibers (Liu et al. 2017) (summarised in Table 2).

8 Pericytes

Considering the affiliation that the MICs have with blood vessels and endothelium, it is unsurprising that intramuscular vasculature is a source of cells that support muscle regeneration. Pericytes are located beneath the basal lamina of small blood vessels and express ALP and NG2. Pericytes are essential for the formation of new blood vessels, being the first cells to invade freshly vascularized tissue (Nehls et al. 1992), where they adopt an angiogenic phenotype (Diaz-Flores et al. 1992) and guide establishment of new blood vessels (Ozerdem and Stallcup 2003). Additionally, pericytes promote endothelial cell survival (Kale et al. 2005), prevent vessel regression (Enge et al. 2002), and are crucial for vessel maturation (Hellström et al. 2001). Angiogenesis is important for muscle regeneration, since only 5 h in an oxygen-deprived environment causes necrosis in up to 90% of surrounding skeletal muscle (Labbe et al. 1987), which can lead to limb amputation where revascularization fails (Conrad et al. 2011).

Two types of pericytes are present in skeletal muscle, and while both type 1 (Nestin-GFP^{-}/NG2^{+}) and type 2 (Nestin-GFP^{+}/NG2^{+}) pericytes are in close proximity to endothelial cells, only type 2 pericytes can express Pax7 (Birbrair et al. 2013b) and contribute to myogenesis (Birbrair et al. 2013a). Pericytes are also capable of differentiating into smooth muscle, adipocytes, or osteoblasts under specific culture conditions but will also spontaneously form multinucleated myotubes if exposed to skeletal muscle differentiation medium. Interestingly, proliferating pericytes do not express MyoD, Pax7, or Myf5, which only appear in terminally differentiated and fused pericytes. Moreover, early postnatal skeletal myogenesis in vivo is supported by pericytes, whose contribution is additionally increased in dystrophic environments or by cardiotoxin injury (Dellavalle et al. 2011). It is of note that involvement of pericytes in myogenesis differs between muscles, for example, being higher in the diaphragm than in the tibialis anterior. Pericytes may also influence maintenance of satellite cell quiescence, as ablation of NG2^{+} pericytes in mouse enhances proliferation of Pax7^{+} cells (Kostallari et al. 2015). An aging microenvironment reduces participation of type 2 pericytes in skeletal muscle regeneration (Birbrair et al. 2013b), although culture in three-dimensional hydrogel rescues some myogenic potential (Fuoco et al. 2014). However, pericytes are also likely to regulate muscle fibrosis. Type 1 pericytes expressing ADAM12, give rise to collagen-producing cells in cases of muscle injury (Dulauroy et al. 2012; Birbrair et al. 2013b) and respond to TGFβ, an essential cytokine for formation of fibrous tissue (Massagué 2012), with increased collagen production in vitro (Birbrair et al. 2013b). However, recently it has been proposed that the multipotency of pericytes in vitro or after transplantation may just arise from the culture conditions (Guimarães-Camboa et al. 2017), which may actually be a useful property for a potential therapeutic cell.

Human pericytes or perivascular cells differ slightly from murine pericytes. These cells are associated with arterioles and capillaries in skeletal muscle and do not express classical myogenic markers such as Myf5, MyoD, myogenin, M-cadherin, nor Pax7

when isolated. Human pericytes do, however, rapidly induce expression of these markers in vitro, or in vivo upon intramuscular transplantation (Crisan et al. 2008).

9 Mesoangioblasts

Mesoangioblast designates a common precursor cell for both vascular and extravascular mesodermal derivatives. Originally isolated as a population of cells from the mouse embryonic dorsal aorta – mesangioblasts express early endothelial and myogenic markers (De Angelis et al. 1999). Freshly isolated murine mesangioblasts express MyoD and Myf5 but retain expression of early endothelial markers including SCA1, CD34, Flk-1, or VE-cadherin in vitro, but not later ones such as vWF. A small proportion (5–30%) additionally express α-SMA, a protein normally expressed in both smooth muscle cells and pericytes (Minasi et al. 2002) and transiently in skeletal myoblasts (Springer et al. 2002). In long-term culture, mesangioblasts lose expression of MyoD and Myf5 and their ability to spontaneously undergo myogenesis but regain myogenic potential if co-cultured with myoblasts (Cossu and Bianco 2003). Aorta-derived myogenic clones lose expression of CD34 during clonal expansion, while endothelial progenitors retain it (Sancricca 2010). Mesoangioblasts isolated from adult murine skeletal muscle have smooth muscle/pericyte markers and have been shown to contribute to skeletal myogenesis during development, regeneration, and upon transplantation (Dellavalle et al. 2011; Tedesco et al. 2010). Human mesangioblasts isolated from skeletal muscle biopsies are similar to pericytes, in that they do not express MyoD, Myf5, myogenin, M-cadherin or NCAM, nor typical endothelial markers CD31, CD34 (unlike murine mesangioblasts), and VEGF2 (Dellavalle et al. 2007; Morosetti et al. 2006). Markers used to isolate and characterize human and murine mesoangioblasts by FACS are given in Table 3.

The ability of mesangioblasts to undergo skeletal myogenesis was also shown by making artificial muscle using human or mouse mesangioblasts to generate multinucleated myotubes in a PEG-Fibrin hydrogel in vitro. Transplantation of such murine mesangioblast/PEG-fibrin hydrogel artificial muscles onto the tibialis anterior resulted in de novo muscle-like tissue, complete with host-derived blood vessels. Indeed, replacement of the murine tibialis anterior with a mesangioblast/hydrogel construct allowed functional recovery – demonstrating the potential for generation of patient-specific replacement muscle if suitable scale-up can be achieved (Fuoco et al. 2015).

Table 3 Marker expression profiles of murine and human mesoangioblasts

Murine mesoangioblasts	Positive	AP, NG2, Sca1, CD34, CD44, CD117, CD140s, CD140b
	Negative	CD31, CD45, CD56, CD133
Human mesoangioblasts	Positive	AP, NG2, CD13, CD44, CD49f, CD90, CD140a, CD140b, CD146
	Negative	CD31, CD34, CD45, CD133, CD56

In pathological settings, intra-arterial delivery of murine mesangioblasts in a limb-girdle muscular dystrophy mouse model (Sampaolesi et al. 2003) or intramuscular injection in *mdx* mice (Berry et al. 2007) increases muscle regeneration. The potential of mesangioblasts to effectively contribute to muscle regeneration in DMD was shown using a microchip engineered model of muscle regeneration. Co-culture of either healthy myoblasts or mesangioblasts with DMD donor-derived myotubes revealed that restoration of dystrophin expression was significantly increased with mesangioblasts (Serena et al. 2016). At least in facioscapulohumeral muscular dystrophy though, human mesangioblasts can vary in their ability to support skeletal muscle regeneration dependent on the characteristics of the muscle from which they are derived (Morosetti et al. 2007, 2011).

Mesoangioblasts have been used in a first-human phase 1/2 clinical trial via intra-arterial delivery for patients suffering from DMD, showing that the procedure is safe, although negligible beneficial effects were seen in muscle function, in part, likely due to the advanced stage of disease at grafting (Cossu et al. 2015). Generally speaking though, mesoangioblasts seem a useful tool for therapy, as they are readily isolated from postnatal tissue, can be extensively expanded in vitro while maintaining their myogenic potential, are easily modifiable to carry therapeutic genes, and are safe for delivery in man (Sancricca 2010).

10 Pluripotent Stem Cells

Differentiation of pluripotent stem cells to skeletal muscle has been achieved with and without genetic manipulation of the pluripotent derivatives. Induction of murine embryonic stem (ES) cells can be achieved by stimulation with DMSO; this however causes differentiation to cardiac, smooth, and skeletal muscle in similar amounts. ES cells would only differentiate specifically to skeletal muscle when simultaneously modified to express MyoD (Dinsmore et al. 1996). Even though transdifferentiation of ES cells into skeletal muscle has been possible for more than 20 years, one of the major difficulties was a low efficiency (Klinger et al. 2003). A way to bypass this efficiency problem is by generation and isolation of mesenchymal precursors from ES cells and subsequent differentiation into skeletal muscle cells (Barberi et al. 2005). Recently, protocols have emerged that allow for direct differentiation of human or murine ES cells first into Pax7-expressing skeletal muscle progenitors and then into mature myocytes (Borchin et al. 2013; Caron et al. 2016; Shelton et al. 2014, 2016; Swartz et al. 2016). Apart from allowing for a higher efficiency in generation of skeletal muscle cells, no genetic manipulation is used in these protocols, making these cells a feasible tool for therapeutic applications, with the caveat of ethical considerations.

Another approach to generate pluripotent stem cells for the subsequent differentiation into skeletal muscle is using inducible pluripotent stem cells (iPSCs). First described by Takahashi and Yamanaka in 2006, iPSCs are mature somatic cells that are reprogrammed to achieve a pluripotent state by the overexpression of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka

2006). IPSCs allow researchers to investigate early developmental processes without the limitations of scarcity of embryonic cells or the ethical concerns associated with the use of embryonic tissue. Importantly, patient cells can be reprogrammed, which has been performed for many conditions, including patients suffering from muscular dystrophies (Tedesco et al. 2012; Smith et al. 2016) and laminopathies (Steele-Stallard et al. 2018), also permitting ex vivo correction of pathogenic mutations (Tedesco et al. 2012). Subsequent differentiation into defined lineages provides powerful insight into pathomechanisms and provides a valuable tool for screening/testing potential therapies (Young et al. 2016). Again, commitment of iPSCs to the myogenic line can be achieved by MyoD overexpression (Maffioletti et al. 2015), but single-cell RNA-seq indicates lack of extracellular cues (insulin) or an inhibitory signaling loop (initialized by overexpressed BMP4) can impede the process (Cacchiarelli et al. 2018). Conditional expression of Pax7 in human ES/iPSCs can direct them to not only make muscle but also allow them to contribute to the myogenic progenitor pool (Darabi et al. 2012), occupying the satellite cell niche and responding to repeated injuries, and so conferring an enduring regenerative potential (Incitti et al. 2019). Indeed, gene expression in such grafted iPSC-derived satellite cells matures in vivo, from their initial embryonic/fetal signature to that more closely resembling postnatal myoblasts (Incitti et al. 2019). Transgene-free protocols are now emerging though, to generate human satellite-like cells and myofibers from iPSC (Chal et al. 2016).

Recently, muscle tissue has been constructed ex vivo from iPSCs (Rao et al. 2018) and now multi-lineage isogenic artificial muscle with myofibers, pericytes, endothelium, and motoneurones all being derived from the same iPSC source (Maffioletti et al. 2018). This artificial muscle was successfully grafted into mouse (Maffioletti et al. 2018), meaning volumetric muscle could be addressed by isogenic artificial muscles derived from the patient's own iPSCs. It will be important to determine how such isogenic artificial muscles perform in an in vivo regenerative setting.

11 Summary

In this brief overview, we have highlighted the range of cells that are able to contribute to the skeletal myogenic lineage. The preeminent resident stem cell of skeletal muscle is the satellite cells, which is essential for successful and complete muscle regeneration, since genetic ablation of $Pax7^{+}$ cells leads to a block in regeneration, with no/few myoblasts detectable in the injured area (Lepper et al. 2011; McCarthy et al. 2011; Relaix and Zammit 2012). In the absence of satellite cells, noncanonical muscle precursors are unable to act as a replacement for repairing muscle. Such non-regenerated muscle tissue is prone to infiltration by inflammatory cells and formation of fibrotic and adipose tissue, showing the importance of prompt and full regeneration.

Satellite cells are not a uniform population though (Biressi and Rando 2010). Recent clustering analysis on single-cell RNA-seq of satellite cells from uninjured

muscle segregates them into multiple populations (Cho and Doles 2017), suggesting that they move along an activation continuum with fluid boundaries, with single-cell mass cytometry (CyTOF) analysis (Porpiglia et al. 2017) also indicating potential subsets. These studies used fluorescent markers to FACs isolate satellite cells, which predefine the population analyzed, and activation will have already been initiated in many of these cells. Gene expression is also dynamic, so such studies provide a snapshot of the mean expression profile of the population at the point of fixation, but new markers will further aid in identification/isolation of satellite cells (Giordani et al. 2019; Hicks et al. 2018; Porpiglia et al. 2017). More recent transcriptomic analysis has used satellite cells fixed much closer to quiescence, allowing more detailed examination of the gene expression profile of quiescent cells (Machado et al. 2017). Employing such methodology at the single-cell level will help further define the nature of satellite cell heterogeneity. Satellite cells remain the most potent cell contributing to myogenesis, and factors including availability, difficulty of isolation, in vitro expansion limitations, loss of potency ex vivo, delivery to patients or difficulty of genetic manipulation are being addressed to enhance their usefulness as therapeutic tools (e.g., Gilbert et al. 2010; Ho et al. 2017). However, alternative myogenic cell types are also being considered and tested for therapy.

At present, the main unorthodox skeletal myogenic progenitors are broadly classed as MICs, those associated with blood vessels and BMDCs. A difficulty in dissecting the contribution of these different cell types to myogenic differentiation lies in their inherent heterogeneity and complex interactions. A panel of markers are normally required for isolation, since many antigens are promiscuous, e.g., Sca1 is found in FAPs and endothelial cells, and CD34 is expressed by both FAPS and satellite cells. Indeed, these populations can overlap, with some PICs, for example, now classed as a subpopulation of FAPs. More subpopulations are being identified as analysis methods are increasingly refined and achieve characterization at an enhanced depth. Single-cell RNA sequencing of mononucleated cells isolated from uninjured muscle shows that cells can be clustered into three major groups: $CD45^+$ blood cells, $CD31^+$ endothelial cells, and Lin^- cells (showing the greatest group diversity) (Giordani et al. 2019) with two newly characterized populations emerging as $Itga7^+/Vcam^-$ or Scx^+ cells.

There may also be a closer relationship between myoblasts and such non-canonical myogenic precursors from in vitro evidence. Some unfused mononucleated cells derived from cultures of multinucleated myotubes derived from immortalized human myoblasts, show a distinct mesenchymal state, with many not expressing myogenin or Myf5, but being positive for ID2 and ID3 (inhibitors of myogenic differentiation) and SPHK1 (Zeng et al. 2016). SPHK1 plays a role in epithelial to mesenchymal transition and expression correlates with invasiveness in cancer cells (Xu et al. 2017). Could transition of some myoblasts to a more mesenchymal state also be an origin for some MICs in skeletal muscle?

Techniques allowing us to characterize cell population at high resolution are constantly evolving, and we can expect that the future will shed more light on the heterogeneity within satellite cells and other cell types with myogenic potential, and the complex cell interactions that govern skeletal muscle regeneration.

Acknowledgment JP is supported by a Wellcome Trust PhD Studentship [203949/Z/16/A]. The Zammit laboratory is supported by the Medical Research Council [MR/P023215/1 and MR/S002472/1], FSH Society Shack Family and Friends research grant [FSHS-82013-06], and Association Française contre les Myopathies [AFM 17865 and 20082].

References

Acharyya S, Butchbach MER, Sahenk Z, Wang H, Saji M, Carathers M, Ringel MD, Skipworth RJE, Fearon KCH, Hollingsworth MA et al (2005) Dystrophin glycoprotein complex dysfunction: a regulatory link between muscular dystrophy and cancer cachexia. Cancer Cell 8:421–432

Agley CC, Rowlerson AM, Velloso CP, Lazarus NL, Harridge SDR (2015) Isolation and quantitative immunocytochemical characterization of primary myogenic cells and fibroblasts from human skeletal muscle. J Vis Exp 95:e52049

Aguennouz M, Vita GL, Messina S, Cama A, Lanzano N, Ciranni A, Rodolico C, Di Giorgio RM, Vita G (2011) Telomere shortening is associated to TRF1 and PARP1 overexpression in Duchenne muscular dystrophy. Neurobiol Aging 32:2190–2197

Allouh MZ, Yablonka-Reuveni Z, Rosser BWC (2008) Pax7 reveals a greater frequency and concentration of satellite cells at the ends of growing skeletal muscle fibers. J Histochem Cytochem 56:77–87

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

Baker JH, Poindextor CE (1991) Muscle regeneration following segmental necrosis in tenotomized muscle fibers. Muscle Nerve 14:348–357

Barberi T, Willis LM, Socci ND, Studer L (2005) Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Med 2:e161

Bareja A, Holt JA, Luo G, Chang C, Lin J, Hinken AC, Freudenberg JM, Kraus WE, Evans WJ, Billin AN (2014) Human and mouse skeletal muscle stem cells: convergent and divergent mechanisms of myogenesis. PLoS One 9:e90398

Baroffio A, Aubry JP, Kaelin A, Krause RM, Hamann M, Bader CR (1993) Purification of human muscle satellite cells by flow cytometry. Muscle Nerve 16:498–505

Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS (2000) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151:1221–1234

Benchaouir R, Meregalli M, Farini A, D'Antona G, Belicchi M, Goyenvalle A, Battistelli M, Bresolin N, Bottinelli R, Garcia L et al (2007) Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell 1:646–657

Berry SE, Liu J, Chaney EJ, Kaufman SJ (2007) Multipotential mesoangioblast stem cell therapy in the mdx/utrn-/- mouse model for Duchenne muscular dystrophy. Regen Med 2:275–288

Besson V, Smeriglio P, Wegener A, Relaix F, Nait Oumesmar B, Sassoon DA, Marazzi G (2011) PW1 gene/paternally expressed gene 3 (PW1/Peg3) identifies multiple adult stem and progenitor cell populations. Proc Natl Acad Sci USA 108:11470–11475

Betsholtz C (2004) Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 15:215–228

Bharathy N, Ling BMT, Taneja R (2013) Epigenetic regulation of skeletal muscle development and differentiation. Subcell Biochem 61:139–150

Birbrair A, Zhang T, Wang Z-M, Messi ML, Enikolopov GN, Mintz A, Delbono O (2013a) Role of pericytes in skeletal muscle regeneration and fat accumulation. Stem Cells Dev 22:2298–2314

Birbrair A, Zhang T, Wang Z-M, Messi ML, Mintz A, Delbono O (2013b) Type-1 pericytes participate in fibrous tissue deposition in aged skeletal muscle. Am J Physiol Cell Physiol 305: C1098–C1113

Biressi S, Rando TA (2010) Heterogeneity in the muscle satellite cell population. Semin Cell Dev Biol 21:845–854

Biressi S, Tagliafico E, Lamorte G, Monteverde S, Tenedini E, Roncaglia E, Ferrari S, Ferrari S, Cusella-De Angelis MG, Tajbakhsh S et al (2007) Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. Dev Biol 304:633–651

Blanco-Bose WE, Yao CC, Kramer RH, Blau HM (2001) Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp Cell Res 265:212–220

Blau HM, Webster C (1981) Isolation and characterization of human muscle cells. Proc Natl Acad Sci USA 78:5623–5627

Blau HM, Webster C, Pavlath GK (1983) Defective myoblasts identified in Duchenne muscular dystrophy. Proc Natl Acad Sci USA 80:4856–4860

Boldrin L, Morgan JE (2012) Human satellite cells: identification on human muscle fibres. PLoS Curr 3:RRN1294

Borchin B, Chen J, Barberi T (2013) Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. Stem Cell Reports 1:620–631

Bosnakovski D, Xu Z, Li W, Thet S, Cleaver O, Perlingeiro RCR, Kyba M (2008) Prospective isolation of skeletal muscle stem cells with a Pax7 reporter. Stem Cells 26:3194–3204

Brazelton TR, Nystrom M, Blau HM (2003) Significant differences among skeletal muscles in the incorporation of bone marrow-derived cells. Dev Biol 262:64–74

Briggs D, Morgan JE (2013) Recent progress in satellite cell/myoblast engraftment - relevance for therapy. FEBS J 280:4281–4293

Cacchiarelli D, Qiu X, Srivatsan S, Manfredi A, Ziller M, Overbey E, Grimaldi A, Grimsby J, Pokharel P, Livak KJ et al (2018) Aligning single-cell developmental and reprogramming trajectories identifies molecular determinants of myogenic reprogramming outcome. Cell Syst 7:258–268.e3

Calhabeu F, Hayashi S, Morgan JE, Relaix F, Zammit PS (2013) Alveolar rhabdomyosarcoma-associated proteins PAX3/FOXO1A and PAX7/FOXO1A suppress the transcriptional activity of MyoD-target genes in muscle stem cells. Oncogene 32:651–662

Cantini M, Giurisato E, Radu C, Tiozzo S, Pampinella F, Senigaglia D, Zaniolo G, Mazzoleni F, Vitiello L (2002) Macrophage-secreted myogenic factors: a promising tool for greatly enhancing the proliferative capacity of myoblasts in vitro and in vivo. Neurol Sci 23:189–194

Caron L, Kher D, Lee KL, McKernan R, Dumevska B, Hidalgo A, Li J, Yang H, Main H, Ferri G et al (2016) A human pluripotent stem cell model of facioscapulohumeral muscular dystrophy-affected skeletal muscles. Stem Cells Transl Med 5:1145–1161

Ceafalan LC, Popescu BO, Hinescu ME (2014) Cellular players in skeletal muscle regeneration. Biomed Res Int 2014:957014

Chal J, Al Tanoury Z, Hestin M, Gobert B, Aivio S, Hick A, Cherrier T, Nesmith AP, Parker KK, Pourquié O (2016) Generation of human muscle fibers and satellite-like cells from human pluripotent stem cells in vitro. Nat Protoc 11:1833–1850

Chargé SBP, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84:209–238

Chazaud B, Brigitte M, Yacoub-Youssef H, Arnold L, Gherardi R, Sonnet C, Lafuste P, Chretien F (2009) Dual and beneficial roles of macrophages during skeletal muscle regeneration. Exerc Sport Sci Rev 37:18–22

Cho DS, Doles JD (2017) Single cell transcriptome analysis of muscle satellite cells reveals widespread transcriptional heterogeneity. Gene 636:54–63

Ciciliot S, Schiaffino S (2010) Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications. Curr Pharm Des 16:906–914

Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122:289–301

Conrad MF, Crawford RS, Hackney LA, Paruchuri V, Abularrage CJ, Patel VI, Lamuraglia GM, Cambria RP (2011) Endovascular management of patients with critical limb ischemia. J Vasc Surg 53:1020–1025

Cornelison DD, Wold BJ (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191:270–283

Cornelison DD, Filla MS, Stanley HM, Rapraeger AC, Olwin BB (2001) Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. Dev Biol 239:79–94

Cosgrove BD, Gilbert PM, Porpiglia E, Mourkioti F, Lee SP, Corbel SY, Llewellyn ME, Delp SL, Blau HM (2014) Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. Nat Med 20:255–264

Cossu G, Bianco P (2003) Mesoangioblasts – vascular progenitors for extravascular mesodermal tissues. Curr Opin Genet Dev 13:537–542

Cossu G, Previtali SC, Napolitano S, Cicalese MP, Tedesco FS, Nicastro F, Noviello M, Roostalu U, Natali Sora MG, Scarlato M et al (2015) Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy. EMBO Mol Med 7:1513–1528

Cottle BJ, Lewis FC, Shone V, Ellison-Hughes GM (2017) Skeletal muscle-derived interstitial progenitor cells (PICs) display stem cell properties, being clonogenic, self-renewing, and multipotent in vitro and in vivo. Stem Cell Res Ther 8:158

Crisan M, Yap S, Casteilla L, Chen C-W, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L et al (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313

Darabi R, Arpke RW, Irion S, Dimos JT, Grskovic M, Kyba M, Perlingeiro RCR (2012) Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. Cell Stem Cell 10:610–619

Daubas P, Tajbakhsh S, Hadchouel J, Primig M, Buckingham M (2000) Myf5 is a novel early axonal marker in the mouse brain and is subjected to post-transcriptional regulation in neurons. Development 127:319–331

Day K, Shefer G, Richardson JB, Enikolopov G, Yablonka-Reuveni Z (2007) Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. Dev Biol 304:246–259

De Angelis L, Berghella L, Coletta M, Lattanzi L, Zanchi M, Cusella-De Angelis MG, Ponzetto C, Cossu G (1999) Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. J Cell Biol 147:869–878

Delbono O (2011) Expression and regulation of excitation-contraction coupling proteins in aging skeletal muscle. Curr Aging Sci 4:248–259

Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, Innocenzi A, Galvez BG, Messina G, Morosetti R et al (2007) Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol 9:255–267

Dellavalle A, Maroli G, Covarello D, Azzoni E, Innocenzi A, Perani L, Antonini S, Sambasivan R, Brunelli S, Tajbakhsh S et al (2011) Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat Commun 2:499

Diaz-Flores L, Gutierrez R, Varela H (1992) Behavior of postcapillary venule pericytes during postnatal angiogenesis. J Morphol 213:33–45

Dinsmore J, Ratliff J, Deacon T, Pakzaban P, Jacoby D, Galpern W, Isacson O (1996) Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. Cell Transplant 5:131–143

Dreyfus PA, Chretien F, Chazaud B, Kirova Y, Caramelle P, Garcia L, Butler-Browne G, Gherardi RK (2004) Adult bone marrow-derived stem cells in muscle connective tissue and satellite cell niches. Am J Pathol 164:773–779

Dulauroy S, Di Carlo SE, Langa F, Eberl G, Peduto L (2012) Lineage tracing and genetic ablation of ADAM12(+) perivascular cells identify a major source of profibrotic cells during acute tissue injury. Nat Med 18:1262–1270

Enge M, Bjarnegård M, Gerhardt H, Gustafsson E, Kalén M, Asker N, Hammes H-P, Shani M, Fässler R, Betsholtz C (2002) Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. EMBO J 21:4307–4316

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 279:1528–1530

Fiore D, Judson RN, Low M, Lee S, Zhang E, Hopkins C, Xu P, Lenzi A, Rossi FMV, Lemos DR (2016) Pharmacological blockage of fibro/adipogenic progenitor expansion and suppression of

regenerative fibrogenesis is associated with impaired skeletal muscle regeneration. Stem Cell Res 17:161–169

Fukada S, Higuchi S, Segawa M, Koda K, Yamamoto Y, Tsujikawa K, Kohama Y, Uezumi A, Imamura M, Miyagoe-Suzuki Y et al (2004) Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. Exp Cell Res 296:245–255

Fukada S, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y, Takeda S'i (2007) Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells 25:2448–2459

Fukada S, Ma Y, Ohtani T, Watanabe Y, Murakami S, Yamaguchi M (2013) Isolation, characterization, and molecular regulation of muscle stem cells. Front Physiol 4:317

Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J (2001) The use of an antifibrosis agent to improve muscle recovery after laceration. Am J Sports Med 29:394–402

Fuoco C, Sangalli E, Vono R, Testa S, Sacchetti B, Latronico MVG, Bernardini S, Madeddu P, Cesareni G, Seliktar D et al (2014) 3D hydrogel environment rejuvenates aged pericytes for skeletal muscle tissue engineering. Front Physiol 5:203

Fuoco C, Rizzi R, Biondo A, Longa E, Mascaro A, Shapira-Schweitzer K, Kossovar O, Benedetti S, Salvatori ML, Santoleri S et al (2015) In vivo generation of a mature and functional artificial skeletal muscle. EMBO Mol Med 7:411–422

Galvez BG, Sampaolesi M, Brunelli S, Covarello D, Gavina M, Rossi B, Constantin G, Torrente Y, Cossu G (2006) Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. J Cell Biol 174:231–243

Garry DJ, Meeson A, Elterman J, Zhao Y, Yang P, Bassel-Duby R, Williams RS (2000) Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. Proc Natl Acad Sci USA 97:5416–5421

Gerli MFM, Moyle LA, Benedetti S, Ferrari G, Ucuncu E, Ragazzi M, Constantinou C, Louca I, Sakai H, Ala P et al (2019) Combined notch and PDGF signaling enhances migration and expression of stem cell markers while inducing perivascular cell features in muscle satellite cells. Stem Cell Reports 12:461–473

Gilbert PM, Havenstrite KL, Magnusson KEG, Sacco A, Leonardi NA, Kraft P, Nguyen NK, Thrun S, Lutolf MP, Blau HM (2010) Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. Science 329:1078–1081

Giordani L, He GJ, Negroni E, Sakai H, Law JYC, Siu MM, Wan R, Corneau A, Tajbakhsh S, Cheung TH et al (2019) High-dimensional single-cell cartography reveals novel skeletal muscle-resident cell populations. Molecular Cell 74:609–621.e6

Glass DJ (2010) Signaling pathways perturbing muscle mass. Curr Opin Clin Nutr Metab Care 13:225–229

Gnocchi VF, White RB, Ono Y, Ellis JA, Zammit PS (2009) Further characterisation of the molecular signature of quiescent and activated mouse muscle satellite cells. PLoS One 4:e5205

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

Goupille O, Pallafacchina G, Relaix F, Conway SJ, Cumano A, Robert B, Montarras D, Buckingham M (2011) Characterization of Pax3-expressing cells from adult blood vessels. J Cell Sci 124:3980–3988

Greising SM, Gransee HM, Mantilla CB, Sieck GC (2012) Systems biology of skeletal muscle: fiber type as an organizing principle. Wiley Interdiscip Rev Syst Biol Med 4:457–473

Guimarães-Camboa N, Cattaneo P, Sun Y, Moore-Morris T, Gu Y, Dalton ND, Rockenstein E, Masliah E, Peterson KL, Stallcup WB et al (2017) Pericytes of multiple organs do not behave as mesenchymal stem cells In Vivo. Cell Stem Cell 20:345–359.e5

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394

Gussoni E, Bennett RR, Muskiewicz KR, Meyerrose T, Nolta JA, Gilgoff I, Stein J, Chan Y, Lidov HG, Bönnemann CG et al (2002) Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. J Clin Invest 110:807–814

Hanson J, Huxley HE (1953) Structural basis of the cross-striations in muscle. Nature 172:530–532
Helal MAM, Shaheen NEM, Abu Zahra FA (2016) Immunomodulatory capacity of the local mesenchymal stem cells transplantation after severe skeletal muscle injury in female rats. Immunopharmacol Immunotoxicol 38:414–422
Hellström M, Gerhardt H, Kalén M, Li X, Eriksson U, Wolburg H, Betsholtz C (2001) Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol 153:543–553
Hernández-Hernández JM, García-González EG, Brun CE, Rudnicki MA (2017) The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. Semin Cell Dev Biol 72:10–18
Heslop L, Morgan JE, Partridge TA (2000) Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J Cell Sci 113(Pt 12):2299–2308
Hicks MR, Hiserodt J, Paras K, Fujiwara W, Eskin A, Jan M, Xi H, Young CS, Evseenko D, Nelson SF et al (2018) ERBB3 and NGFR mark a distinct skeletal muscle progenitor cell in human development and hPSCs. Nat Cell Biol 20:46–57
Ho ATV, Palla AR, Blake MR, Yucel ND, Wang YX, Magnusson KEG, Holbrook CA, Kraft PE, Delp SL, Blau HM (2017) Prostaglandin E2 is essential for efficacious skeletal muscle stem-cell function, augmenting regeneration and strength. Proc Natl Acad Sci 114(26):6675–6684
Hogendoorn S, Duijnisveld BJ, van Duinen SG, Stoel BC, van Dijk JG, Fibbe WE, Nelissen RGHH (2014) Local injection of autologous bone marrow cells to regenerate muscle in patients with traumatic brachial plexus injury: a pilot study. Bone Joint Res 3:38–47
Ikemoto M, Fukada S-I, Uezumi A, Masuda S, Miyoshi H, Yamamoto H, Wada MR, Masubuchi N, Miyagoe-Suzuki Y, Takeda S'i (2007) Autologous transplantation of SM/C-2.6(+) satellite cells transduced with micro-dystrophin CS1 cDNA by lentiviral vector into mdx mice. Mol Ther 15:2178–2185
Incitti T, Magli A, Darabi R, Yuan C, Lin K, Arpke RW, Azzag K, Yamamoto A, Stewart R, Thomson JA, et al (2019) Pluripotent stem cell-derived myogenic progenitors remodel their molecular signature upon in vivo engraftment. Proc Natl Acad Sci 116(10):4346–4351
Irintchev A, Zeschnigk M, Starzinski-Powitz A, Wernig A (1994) Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev Dyn 199:326–337
Jackson KA, Mi T, Goodell MA (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci USA 96:14482–14486
Janssen I, Heymsfield SB, Wang ZM, Ross R (2000) Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. J Appl Physiol (1985) 89:81–88
Järvinen TAH, Järvinen TLN, Kääriäinen M, Kalimo H, Järvinen M (2005) Muscle injuries: biology and treatment. Am J Sports Med 33:745–764
Joe AWB, Yi L, Natarajan A, Le Grand F, So L, Wang J, Rudnicki MA, Rossi FMV (2010) Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. Nat Cell Biol 12:153–163
Jørgensen LH, Jensen CH, Wewer UM, Schrøder HD (2007) Transgenic overexpression of ADAM12 suppresses muscle regeneration and aggravates dystrophy in aged mdx mice. Am J Pathol 171:1599–1607
Kakulas BA, Papadimitriou JM, Knight JO, Mastaglia FL (1968) Normal and abnormal human muscle in tissue culture. Proc Aust Assoc Neurol 5:79–85
Kale S, Hanai J, Chan B, Karihaloo A, Grotendorst G, Cantley L, Sukhatme VP (2005) Microarray analysis of in vitro pericyte differentiation reveals an angiogenic program of gene expression. FASEB J 19:270–271
Katz B (1961) The termination of the afferent nerve fibre in the muscle spindle of the frog. Philos Trans R Soc Lond Ser B Biol Sci 243:221–240
Klinger F-G, Scaldaferri M-L, Di Carlo A, Baiocchi M, Coletta M, Cossu G, De Felici M (2003) Myogenic potential of mouse primordial germ cells. Int J Dev Biol 47:303–305
Kobari L, Giarratana MC, Pflumio F, Izac B, Coulombel L, Douay L (2001) CD133+ cell selection is an alternative to CD34+ cell selection for ex vivo expansion of hematopoietic stem cells. J Hematother Stem Cell Res 10:273–281

Kostallari E, Baba-Amer Y, Alonso-Martin S, Ngoh P, Relaix F, Lafuste P, Gherardi RK (2015) Pericytes in the myovascular niche promote post-natal myofiber growth and satellite cell quiescence. Development 142:1242–1253

Kowalski K, Dos Santos M, Maire P, Ciemerych MA, Brzoska E (2018) Induction of bone marrow-derived cells myogenic identity by their interactions with the satellite cell niche. Stem Cell Res Ther 9:258

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

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

Labbe R, Lindsay T, Walker PM (1987) The extent and distribution of skeletal muscle necrosis after graded periods of complete ischemia. J Vasc Surg 6:152–157

Lapidos KA, Chen YE, Earley JU, Heydemann A, Huber JM, Chien M, Ma A, McNally EM (2004) Transplanted hematopoietic stem cells demonstrate impaired sarcoglycan expression after engraftment into cardiac and skeletal muscle. J Clin Invest 114:1577–1585

Latil M, Rocheteau P, Châtre L, Sanulli S, Mémet S, Ricchetti M, Tajbakhsh S, Chrétien F (2012) Skeletal muscle stem cells adopt a dormant cell state post mortem and retain regenerative capacity. Nat Commun 3:903

Lepper C, Partridge TA, Fan C-M (2011) An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Development 138:3639–3646

Linard C, Brachet M, L'homme B, Strup-Perrot C, Busson E, Bonneau M, Lataillade J-J, Bey E, Benderitter M (2018) Long-term effectiveness of local BM-MSCs for skeletal muscle regeneration: a proof of concept obtained on a pig model of severe radiation burn. Stem Cell Res Ther 9:299

Lindström M, Thornell L-E (2009) New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. Histochem Cell Biol 132:141–157

Liu N, Garry GA, Li S, Bezprozvannaya S, Sanchez-Ortiz E, Chen B, Shelton JM, Jaichander P, Bassel-Duby R, Olson EN (2017) A Twist2-dependent progenitor cell contributes to adult skeletal muscle. Nat Cell Biol 19:202–213

Luth ES, Jun SJ, Wessen MK, Liadaki K, Gussoni E, Kunkel LM (2008) Bone marrow side population cells are enriched for progenitors capable of myogenic differentiation. J Cell Sci 121:1426–1434

Luz MAM, Marques MJ, Santo Neto H (2002) Impaired regeneration of dystrophin-deficient muscle fibers is caused by exhaustion of myogenic cells. Braz J Med Biol Res 35:691

Machado L, Esteves de Lima J, Fabre O, Proux C, Legendre R, Szegedi A, Varet H, Ingerslev LR, Barrès R, Relaix F et al (2017) In situ fixation redefines quiescence and early activation of skeletal muscle stem cells. Cell Rep 21:1982–1993

Maffioletti SM, Gerli MFM, Ragazzi M, Dastidar S, Benedetti S, Loperfido M, VandenDriessche T, Chuah MK, Tedesco FS (2015) Efficient derivation and inducible differentiation of expandable skeletal myogenic cells from human ES and patient-specific iPS cells. Nat Protoc 10:941–958

Maffioletti SM, Sarcar S, Henderson ABH, Mannhardt I, Pinton L, Moyle LA, Steele-Stallard H, Cappellari O, Wells KE, Ferrari G et al (2018) Three-dimensional human iPSC-derived artificial skeletal muscles model muscular dystrophies and enable multilineage tissue engineering. Cell Rep 23:899–908

Marg A, Escobar H, Gloy S, Kufeld M, Zacher J, Spuler A, Birchmeier C, Izsvák Z, Spuler S (2014) Human satellite cells have regenerative capacity and are genetically manipulable. J Clin Invest 124:4257–4265

Massagué J (2012) TGFβ signalling in context. Nat Rev Mol Cell Biol 13:616–630

Mauro A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493–495

McCarthy JJ, Mula J, Miyazaki M, Erfani R, Garrison K, Farooqui AB, Srikuea R, Lawson BA, Grimes B, Keller C et al (2011) Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. Development 138:3657–3666

Meng J, Muntoni F, Morgan JE (2011) Stem cells to treat muscular dystrophies – Where are we? Neuromuscul Disord 21:4–12

Meng J, Chun S, Asfahani R, Lochmüller H, Muntoni F, Morgan J (2014) Human skeletal muscle-derived CD133(+) cells form functional satellite cells after intramuscular transplantation in immunodeficient host mice. Mol Ther 22:1008–1017

Meng J, Muntoni F, Morgan J (2018) CD133+ cells derived from skeletal muscles of Duchenne muscular dystrophy patients have a compromised myogenic and muscle regenerative capability. Stem Cell Res 30:43–52

Minasi MG, Riminucci M, De Angelis L, Borello U, Berarducci B, Innocenzi A, Caprioli A, Sirabella D, Baiocchi M, De Maria R et al (2002) The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development 129:2773–2783

Mintz B, Baker WW (1967) Normal mammalian muscle differentiation and gene control of isocitrate dehydrogenase synthesis. Proc Natl Acad Sci USA 58:592–598

Mitchell KJ, Pannérec 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

Moncaut N, Rigby PWJ, Carvajal JJ (2013) Dial M(RF) for myogenesis. FEBS J 280:3980–3990

Montanaro F, Liadaki K, Schienda J, Flint A, Gussoni E, Kunkel LM (2004) Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. Exp Cell Res 298:144–154

Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, Buckingham M (2005) Direct isolation of satellite cells for skeletal muscle regeneration. Science 309:2064–2067

Morosetti R, Mirabella M, Gliubizzi C, Broccolini A, De Angelis L, Tagliafico E, Sampaolesi M, Gidaro T, Papacci M, Roncaglia E et al (2006) MyoD expression restores defective myogenic differentiation of human mesoangioblasts from inclusion-body myositis muscle. Proc Natl Acad Sci USA 103:16995–17000

Morosetti R, Mirabella M, Gliubizzi C, Broccolini A, Sancricca C, Pescatori M, Gidaro T, Tasca G, Frusciante R, Tonali PA et al (2007) Isolation and characterization of mesoangioblasts from facioscapulohumeral muscular dystrophy muscle biopsies. Stem Cells 25:3173–3182

Morosetti R, Gidaro T, Broccolini A, Gliubizzi C, Sancricca C, Tonali PA, Ricci E, Mirabella M (2011) Mesoangioblasts from facioscapulohumeral muscular dystrophy display in vivo a variable myogenic ability predictable by their in vitro behavior. Cell Transplant 20:1299–1313

Motohashi N, Asakura Y, Asakura A (2014) Isolation, culture, and transplantation of muscle satellite cells. J Vis Exp e50846. https://doi.org/10.3791/50846

Moyle LA, Zammit PS (2014) Isolation, culture and immunostaining of skeletal muscle fibres to study myogenic progression in satellite cells. Methods Mol Biol 1210:63–78

Naldaiz-Gastesi N, Goicoechea M, Alonso-Martín S, Aiastui A, López-Mayorga M, García-Belda P, Lacalle J, San José C, Araúzo-Bravo MJ, Trouilh L et al (2016) Identification and characterization of the dermal panniculus carnosus muscle stem cells. Stem Cell Reports 7:411–424

Negroni E, Riederer I, Chaouch S, Belicchi M, Razini P, Di Santo J, Torrente Y, Butler-Browne GS, Mouly V (2009) In vivo myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. Mol Ther 17:1771–1778

Negroni E, Bigot A, Butler-Browne GS, Trollet C, Mouly V (2016) Cellular therapies for muscular dystrophies: frustrations and clinical successes. Hum Gene Ther 27:117–126

Negroni E, Vallese D, Vilquin JT, Butler-Browne G, Mouly V, Trollet C (2011) Current advances in cell therapy strategies for muscular dystrophies. Expert Opin Biol Ther 11:157–76

Nehls V, Denzer K, Drenckhahn D (1992) Pericyte involvement in capillary sprouting during angiogenesis in situ. Cell Tissue Res 270:469–474

Ontell M, Hughes D, Bourke D (1982) Secondary myogenesis of normal muscle produces abnormal myotubes. Anat Rec 204:199–207

Ortuste Quiroga HP, Goto K, Zammit PS (2016) Isolation, cryosection and immunostaining of skeletal muscle. Methods Mol Biol 1460:85–100

Ozerdem U, Stallcup WB (2003) Early contribution of pericytes to angiogenic sprouting and tube formation. Angiogenesis 6:241–249

Pannerec A, Formicola L, Besson V, Marazzi G, Sassoon DA (2013) Defining skeletal muscle resident progenitors and their cell fate potentials. Development 140:2879–2891

Pawlikowski B, Lee L, Zuo J, Kramer RH (2009) Analysis of human muscle stem cells reveals a differentiation-resistant progenitor cell population expressing Pax7 capable of self-renewal. Dev Dyn 238:138–149

Pelagiadis I, Relakis K, Kalmanti L, Dimitriou H (2012) CD133 immunomagnetic separation: effectiveness of the method for CD133(+) isolation from umbilical cord blood. Cytotherapy 14:701–706

Périé S, Trollet C, Mouly V, Vanneaux V, Mamchaoui K, Bouazza B, Marolleau JP, Laforêt P, Chapon F, Eymard B et al (2014) Autologous myoblast transplantation for oculopharyngeal muscular dystrophy: a phase I/IIa clinical study. Mol Ther 22:219–225

Pette D, Spamer C (1986) Metabolic properties of muscle fibers. Fed Proc 45:2910–2914

Pisani DF, Clement N, Loubat A, Plaisant M, Sacconi S, Kurzenne J-Y, Desnuelle C, Dani C, Dechesne CA (2010) Hierarchization of myogenic and adipogenic progenitors within human skeletal muscle. Stem Cells 28:2182–2194

Pisconti A, Bernet JD, Olwin BB (2012) Syndecans in skeletal muscle development, regeneration and homeostasis. Muscles Ligaments Tendons J 2:1–9

Pogogeff IA, Murray MR (1946) Form and behavior of adult mammalian skeletal muscle in vitro. Anat Rec 95:321–335

Porpiglia E, Samusik N, Van Ho AT, Cosgrove BD, Mai T, Davis KL, Jager A, Nolan GP, Bendall SC, Fantl WJ et al (2017) High-resolution myogenic lineage mapping by single-cell mass cytometry. Nat Cell Biol 19:558–567

Quarta M, Brett JO, DiMarco R, De Morree A, Boutet SC, Chacon R, Gibbons MC, Garcia VA, Su J, Shrager JB et al (2016) An artificial niche preserves the quiescence of muscle stem cells and enhances their therapeutic efficacy. Nat Biotechnol 34:752–759

Rao L, Qian Y, Khodabukus A, Ribar T, Bursac N (2018) Engineering human pluripotent stem cells into a functional skeletal muscle tissue. Nat Commun 9:126

Reimann J, Brimah K, Schroder R, Wernig A, Beauchamp JR, Partridge TA (2004) Pax7 distribution in human skeletal muscle biopsies and myogenic tissue cultures. Cell Tissue Res 315:233–242

Relaix F, Zammit PS (2012) Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Develepment 139:2845–2856

Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham M (2006) Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 172:91–102

Richler C, Yaffe D (1970) The in vitro cultivation and differentiation capacities of myogenic cell lines. Dev Biol 23:1–22

Rivier F (2004) Role of bone marrow cell trafficking in replenishing skeletal muscle SP and MP cell populations. J Cell Sci 117:1979–1988

Rosen GD, Sanes JR, LaChance R, Cunningham JM, Roman J, Dean DC (1992) Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. Cell 69:1107–1119

Rosenblatt JD (1992) A time course study of the isometric contractile properties of rat extensor digitorum longus muscle injected with bupivacaine. Comp Biochem Physiol Comp Physiol 101:361–367

Saini J, McPhee JS, Al-Dabbagh S, Stewart CE, Al-Shanti N (2016) Regenerative function of immune system: Modulation of muscle stem cells. Ageing Res Rev 27:67–76

Sambasivan R, Gayraud-Morel B, Dumas G, Cimper C, Paisant S, Kelly RG, Tajbakhsh S (2009) Distinct regulatory cascades govern extraocular and pharyngeal arch muscle progenitor cell fates. Dev Cell 16:810–821

Sampaolesi M, Torrente Y, Innocenzi A, Tonlorenzi R, D'Antona G, Pellegrino MA, Barresi R, Bresolin N, De Angelis MGC, Campbell KP et al (2003) Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. Science 301:487–492

Sancricca C (2010) Vessel-associated stem cells from skeletal muscle: from biology to future uses in cell therapy. World J Stem Cells 2:39

Scharner J, Zammit PS (2011) The muscle satellite cell at 50: the formative years. Skelet Muscle 1:28

Schmalbruch H (1976) The morphology of regeneration of skeletal muscles in the rat. Tissue Cell 8:673–692
Schultz E, Gibson MC, Champion T (1978) Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. J Exp Zool 206:451–456
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 102:777–786
Serena E, Zatti S, Zoso A, Lo Verso F, Tedesco FS, Cossu G, Elvassore N (2016) Skeletal muscle differentiation on a chip shows human donor mesoangioblasts' efficiency in restoring dystrophin in a duchenne muscular dystrophy model. Stem Cells Transl Med 5:1676
Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z (2006) Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol 294:50–66
Shefer G, Rauner G, Yablonka-Reuveni Z, Benayahu D (2010) Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. PLoS One 5:e13307
Shelton M, Metz J, Liu J, Carpenedo RL, Demers S-P, Stanford WL, Skerjanc IS (2014) Derivation and expansion of PAX7-positive muscle progenitors from human and mouse embryonic stem cells. Stem Cell Reports 3:516–529
Shelton M, Kocharyan A, Liu J, Skerjanc IS, Stanford WL (2016) Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. Methods 101:73–84
Shen W, Li Y, Tang Y, Cummins J, Huard J (2005) NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. Am J Pathol 167:1105–1117
Sherwood RI, Christensen JL, Conboy IM, Conboy MJ, Rando TA, Weissman IL, Wagers AJ (2004) Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell 119:543–554
Sinha-Hikim I, Taylor WE, Gonzalez-Cadavid NF, Zheng W, Bhasin S (2004) Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. J Clin Endocrinol Metab 89:5245–5255
Smith AST, Davis J, Lee G, Mack DL, Kim D-H (2016) Muscular dystrophy in a dish: engineered human skeletal muscle mimetics for disease modeling and drug discovery. Drug Discov Today 21:1387
Springer ML, Ozawa CR, Blau HM (2002) Transient production of ?-smooth muscle actin by skeletal myoblasts during differentiation in culture and following intramuscular implantation. Cell Motil Cytoskeleton 51:177–186
Steele-Stallard HB, Pinton L, Sarcar S, Ozdemir T, Maffioletti SM, Zammit PS, Tedesco FS (2018) Modeling skeletal muscle laminopathies using human induced pluripotent stem cells carrying pathogenic LMNA mutations. Front Physiol 9:1332
Strömberg A, Jansson M, Fischer H, Rullman E, Hägglund H, Gustafsson T (2013) Bone marrow derived cells in adult skeletal muscle tissue in humans. Skelet Muscle 3:12
Stumm J, Vallecillo-García P, Vom Hofe-Schneider S, Ollitrault D, Schrewe H, Economides AN, Marazzi G, Sassoon DA, Stricker S (2018) Odd skipped-related 1 (Osr1) identifies muscle-interstitial fibro-adipogenic progenitors (FAPs) activated by acute injury. Stem Cell Res 32:8–16
Swartz EW, Baek J, Pribadi M, Wojta KJ, Almeida S, Karydas A, Gao F-B, Miller BL, Coppola G (2016) A novel protocol for directed differentiation of C9orf72-associated human induced pluripotent stem cells into contractile skeletal myotubes. Stem Cells Transl Med 5:1461
Tajbakhsh S, Bober E, Babinet C, Pournin S, Arnold H, Buckingham M (1996) Gene targeting the myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fibres as well as early embryonic muscle. Dev Dyn 206:291–300
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takano H, Komuro I, Oka T, Shiojima I, Hiroi Y, Mizuno T, Yazaki Y (1998) The Rho family G proteins play a critical role in muscle differentiation. Mol Cell Biol 18:1580–1589

Tamaki T, Akatsuka A, Yoshimura S, Roy RR, Edgerton VR (2002) New fiber formation in the interstitial spaces of rat skeletal muscle during postnatal growth. J Histochem Cytochem 50:1097–1111

Tamaki T, Akatsuka A, Okada Y, Matsuzaki Y, Okano H, Kimura M (2003) Growth and differentiation potential of main- and side-population cells derived from murine skeletal muscle. Exp Cell Res 291:83–90

Tanaka KK, Hall JK, Troy AA, Cornelison DDW, Majka SM, Olwin BB (2009) Syndecan-4-expressing muscle progenitor cells in the SP engraft as satellite cells during muscle regeneration. Cell Stem Cell 4:217–225

Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G (2010) Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. J Clin Invest 120:11–19

Tedesco FS, Gerli MFM, Perani L, Benedetti S, Ungaro F, Cassano M, Antonini S, Tagliafico E, Artusi V, Longa E et al (2012) Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy. Sci Transl Med 4:140ra89–140ra89

Tierney MT, Sacco A (2016) Satellite cell heterogeneity in skeletal muscle homeostasis. Trends Cell Biol 26:434–444

Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G, Tonlorenzi R, Porretti L, Gavina M, Mamchaoui K et al (2004) Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest 114:182–195

Torrente Y, Belicchi M, Marchesi C, D'Antona G, Cogiamanian F, Pisati F, Gavina M, Giordano R, Tonlorenzi R, Fagiolari G et al (2007) Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients. Cell Transplant 16:563–577

Uezumi A, Fukada S, Yamamoto N, Takeda S'i, Tsuchida K (2010) Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. Nat Cell Biol 12:143–152

Uezumi A, Ito T, Morikawa D, Shimizu N, Yoneda T, Segawa M, Yamaguchi M, Ogawa R, Matev MM, Miyagoe-Suzuki Y et al (2011) Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. J Cell Sci 124:3654–3664

van Velthoven CTJ, de Morree A, Egner IM, Brett JO, Rando TA (2017) Transcriptional profiling of quiescent muscle stem cells in vivo. Cell Rep 21:1994–2004

Vilquin J-T, Catelain C, Vauchez K (2011) Cell therapy for muscular dystrophies: advances and challenges. Curr Opin Organ Transplant 16:640–649

Volonte D, Liu Y, Galbiati F (2005) The modulation of caveolin-1 expression controls satellite cell activation during muscle repair. FASEB J 19:237–239

Walsh FS, Ritter MA (1981) Surface antigen differentiation during human myogenesis in culture. Nature 289:60–64

Weintraub H (1993) The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell 75:1241–1244

Wernig G, Janzen V, Schäfer R, Zweyer M, Knauf U, Hoegemeier O, Mundegar RR, Garbe S, Stier S, Franz T et al (2005) The vast majority of bone-marrow-derived cells integrated into mdx muscle fibers are silent despite long-term engraftment. Proc Natl Acad Sci USA 102:11852–11857

Whalen RG, Harris JB, Butler-Browne GS, Sesodia S (1990) Expression of myosin isoforms during notexin-induced regeneration of rat soleus muscles. Dev Biol 141:24–40

Xu X, Wilschut KJ, Kouklis G, Tian H, Hesse R, Garland C, Sbitany H, Hansen S, Seth R, Knott PD et al (2015) Human satellite cell transplantation and regeneration from diverse skeletal muscles. Stem Cell Reports 5:419–434

Xu C-Y, Liu S-Q, Qin M-B, Zhuge C-F, Qin L, Qin N, Lai M-Y, Huang J-A (2017) SphK1 modulates cell migration and EMT-related marker expression by regulating the expression of p-FAK in colorectal cancer cells. Int J Mol Med 39:1277–1284

Yablonka-Reuveni Z (2011) The skeletal muscle satellite cell: still young and fascinating at 50. J Histochem Cytochem 59:1041–1059

Yamaguchi M, Ogawa R, Watanabe Y, Uezumi A, Miyagoe-Suzuki Y, Tsujikawa K, Yamamoto H, Takeda S, Fukada S (2012) Calcitonin receptor and Odz4 are differently expressed in Pax7-positive cells during skeletal muscle regeneration. J Mol Histol 43:581–587

Yao Y, Norris EH, Mason EC, Strickland S (2016) Laminin regulates PDGFRβ+ cell stemness and muscle development. Nat Commun 7:11415

Yin H, Price F, Rudnicki MA (2013) Satellite cells and the muscle stem cell niche. Physiol Rev 93:23–67

Young CS, Hicks MR, Ermolova NV, Nakano H, Jan M, Younesi S, Karumbayaram S, Kumagai-Cresse C, Wang D, Zack JA, Kohn DB, Nakano A, Nelson SF, Miceli MC, Spencer MJ, Pyle AD (2016) A single CRISPR-cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. Cell Stem Cell 18:533–540

Zammit PS (2008) All muscle satellite cells are equal, but are some more equal than others? J Cell Sci 121:2975–2982

Zammit PS (2017) Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. Semin Cell Dev Biol 72:19–32

Zammit PS, Heslop L, Hudon V, Rosenblatt JD, Tajbakhsh S, Buckingham ME, Beauchamp JR, Partridge TA (2002) Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Exp Cell Res 281:39–49

Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166:347–357

Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, Beauchamp JR (2006) Pax7 and myogenic progression in skeletal muscle satellite cells. J Cell Sci 119:1824–1832

Zeng W, Jiang S, Kong X, El-Ali N, Ball AR, Ma CI-H, Hashimoto N, Yokomori K, Mortazavi A (2016) Single-nucleus RNA-seq of differentiating human myoblasts reveals the extent of fate heterogeneity. Nucleic Acids Res 44:e158. gkw739

Zheng B, Cao B, Crisan M, Sun B, Li G, Logar A, Yap S, Pollett JB, Drowley L, Cassino T et al (2007) Prospective identification of myogenic endothelial cells in human skeletal muscle. Nat Biotechnol 25:1025–1034

Zuba-Surma EK, Kucia M, Ratajczak J, Ratajczak MZ (2009) “Small stem cells” in adult tissues: very small embryonic-like stem cells stand up! Cytom Part A 75:4–13

Cells for Cartilage Regeneration

Gerjo J. V. M. van Osch, Andrea Barbero, Mats Brittberg, Diego Correa, Solvig Diederichs, Mary B. Goldring, Tim Hardingham, Florien Jenner, Roberto Narcisi, Mark A. Randolph, and Sylvia Nuernberger

Contents

G. J. V. M. van Osch (✉)
Department of Orthopaedics and Department of Otorhinolaryngology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
e-mail: g.vanosch@erasmusmc.nl

A. Barbero
Department of Biomedicine, University of Basel and University Hospital of Basel, Basel, Switzerland
e-mail: andrea.barbero@usb.ch

M. Brittberg
Cartilage Research Unit, University of Gothenburg, Region Halland Orthopaedics, Kungsbacka Hospital, Kungsbacka, Sweden
e-mail: mats.brittberg@telia.com

D. Correa
Department of Orthopaedics, Division of Sports Medicine, Diabetes Research Institute, Cell Transplant Center, University of Miami, Miller School of Medicine, Miami, FL, USA

Department of Biology, Skeletal Research Center, Case Western Reserve University, Cleveland, OH, USA
e-mail: dxc821@miami.edu

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_1

S. Diederichs
Research Center for Experimental Orthopaedics, Heidelberg University Hospital, Heidelberg, Germany
e-mail: solvig.diederichs@med.uni-heidelberg.de

M. B. Goldring
Hospital for Special Surgery and Weill Cornell Medical College, HSS Research Institute, New York, NY, USA
e-mail: goldringm@hss.edu

T. Hardingham
Wellcome Centre for Cell-Matrix Research, Division of Cell-Matrix Biology and Regenerative Medicine, School of Biology, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK
e-mail: tim.hardingham@manchester.ac.uk

F. Jenner
University Equine Hospital, University of Veterinary Medicine Vienna, Vienna, Austria
e-mail: Florien.Jenner@vetmeduni.ac.at

R. Narcisi
Department of Orthopaedics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
e-mail: r.narcisi@erasmusmc.nl

M. A. Randolph
Harvard Medical School, Massachusetts General Hospital, Boston, MA, USA
e-mail: marandolph@mgh.harvard.edu

S. Nuernberger
Department of Trauma Surgery, Medical University of Vienna, Vienna, Austria

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

Abstract

Cartilage, at all sites of our body, has limited intrinsic capacity to repair. There are numerous methods to use when repairing cartilage, and the preferred method depends on the location of the cartilage defect, whether it is the nose, the ear, the airway, or a certain site in the joint. Different cell types with chondrogenic capacities are being used for cartilage regeneration. In this chapter we discuss the expansion in culture, the chondrogenic capacity, and the (clinical) application of articular chondrocytes, nasal chondrocytes, auricular chondrocytes, chondrocyte cell lines, mesenchymal stem cells, and pluripotent stem cells.

1 Introduction

Cartilage defect biology is strongly related to the tissue's avascularity, lack of innervation, and high matrix to cell ratio. These conditions prevent cartilage regeneration due to insufficient numbers of intrinsic chondrocytes or alternative cell sources that can migrate into the defect and fill it with matrix. In those cartilages with a covering perichondrium with progenitor cells, this may facilitate a certain degree of regeneration; however, larger defects due to trauma, surgical removal of a tumor, or congenital malformations (microtia) require other replacement strategies. Articular cartilage, which lacks a perichondrium, has especially poor prognosis in terms of regeneration and requires clinical intervention, even in case of small defects.

Tissue engineering aims to effectively regenerate the tissue and achieve its specific properties and function and not just repair with scar tissue formation. While nasal and auricular cartilages have their major function in providing shape and elastic deformation, articular cartilage has the specialized function of load bearing. The morphological and biomechanical properties of cartilage are related to these specific requirements and distinguish three subtypes: hyaline, elastic, and fibrous cartilage. All three are composed of a large amount of extracellular matrix (ECM) in which major components are the marker protein collagen type II and the aggrecan core protein with its attached glycosaminoglycans (GAGs), in which are embedded the cells of a distinct mesenchymal lineage, the chondrocytes. Cartilage tissues in the various locations differ in detailed matrix composition (relative quantities and presence of additional matrix proteins) and matrix organization as

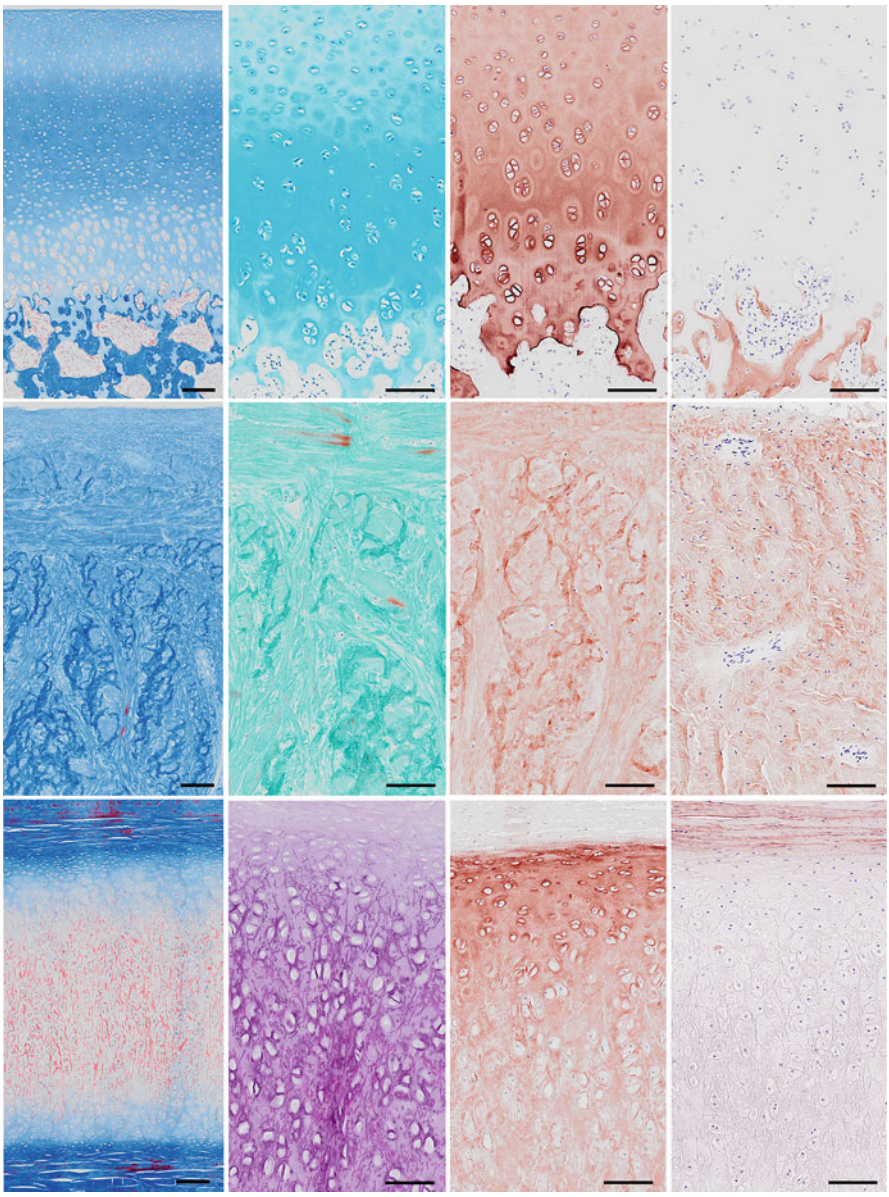


Fig. 1 Histochemical and immunohistochemical stainings characterizing hyaline, fibrous, and elastic bovine cartilage. The hyaline articular cartilage (1st line) has a dense matrix with cells homogeneously distributed cells but with a certain zonation discernible by cell density, cell orientation, and matrix density. Fibrous cartilage (meniscus, 2nd line) is composed of large bundles stretching crosswise through the meniscus and along the outer tissue surface. Ear cartilage (3rd line) contains elastic fibers forming a network around the cells and stretching through tissue ending in an outer area free of elastic fibers and the perichondrium. The *very left images* are MSB (= Martius Scarlet Blue) stainings displaying collagen in blue and cells and elastic fibers in red. In the *second*

well as chondrocyte subtypes. Hyaline cartilage has the highest concentration of GAGs in which are embedded the dense network of collagen fibrils (Fig. 1a). In fibrous cartilage the collagen bundles are aligned along the stress axis, either parallel or at an oblique angle, in, for example, the meniscus (Fig. 1b). In elastic cartilage the collagen fibrils are arranged around the cells and stretch throughout the tissue, accompanied by elastic fiber (Fig. 1c).

Chondrocytes are the cells that regulate the matrix turnover. They have a low mitotic rate and partially anaerobic metabolism, which allows them to persist in the dense non-vascularized matrix. There are different subtypes of chondrocytes, either within a cartilage type (from the surface to the depth) or between cartilage types (articular versus nasal). In addition to structural-functional reasons, differences are related to their ontogenetic origin. During embryogenesis chondrocytes arise from mesenchymal progenitors descending from three distinct embryonic cell lineages of mesodermal and mesoectodermal origin: cranial neural crest cells form the majority of the craniofacial skeleton; mesodermal paraxial mesoderm is responsible for rib, vertebral joint, intervertebral disc, plus vertebral body formation; and lateral plate mesoderm gives rise to the appendicular skeleton and the sternum (Glenister 1976; Olsen et al. 2000; McIntyre et al. 2007). The mesenchymal chondroprogenitor cells undergo chondrogenesis, a multistep cascade under a tight spatiotemporal control, encompassing cell recruitment, migration along specific pathways to their target locations, proliferation, condensation into skeletal primordia, and differentiation into chondrocytes (Hall and Miyake 2000; DeLise et al. 2000; Goldring et al. 2006). Chondrocytes then produce extracellular cartilage matrix with biochemical, physical, and biomechanical properties specific for their anatomical location, which can be classified as hyaline, elastic, or fibrous cartilage (Quinn et al. 2005; Isogai et al. 2006; Somoza et al. 2014). Elastic cartilage, which is found in the scaffold for the epiglottis, the Eustachian tube, and the external ear among others, contains networks of elastic fibers throughout the matrix, rendering it stiff but elastic. Fibrocartilage contains largely collagen I and is located in areas subjected predominantly to tensile stress, such as in the intervertebral discs and menisci. Hyaline cartilage is rich in collagen II and hydrophilic proteoglycans, making it highly resistant to compressive load and shear forces. Cartilage forms the skeletal anlagen during embryogenesis, the postnatal growth plate, the ventral ribs, the nasal septum, the larynx, the trachea, and the smooth articular surfaces in diarthrodial joints.

Cartilage can also be classified based on the phenotypic stability of the chondrocytes and the nature of the cartilage matrix itself as permanent or transient

Fig. 1 (continued) column, special stainings reveal the specific characteristics of the tissue: Alcian blue stains glycosaminoglycans in hyaline articular cartilage in blue, Masson trichrome stains collagen in the fibrous meniscus in green, and orcein displays the network of elastic fibers. Immunohistochemical images in the *third column* demonstrate the distribution of the marker protein collagen type II and those in the *very right column* collagen type I in fibrocartilage and the surrounding tissues of articular and nasal cartilage. Images of different stainings were taken from the same areas, except for fibrocartilage where collagen type I was most obvious in the outer and type II stainings in the inner region of the meniscus

cartilage. Cartilage responsible for bone formation, growth, and repair, such as the epiphyseal growth plate of long bones and the cartilaginous callus formed during endochondral fracture healing, is composed of transient chondrocytes (reviewed by Kirsch et al. 2000; Iwamoto et al. 2001, 2005; Lefebvre and Smits 2005). Transient chondrocytes undergo maturation and terminal differentiation and provide the environment for endochondral ossification and for replacement by bone and marrow cells. In contrast, permanent cartilage of articular surfaces, airways, ears, and nose persists throughout life.

Since tissue regeneration at its best recapitulates embryonic tissue morphogenesis, a better understanding of cartilage lineage divergence, the various chondrocyte progenitor population, and the gene expression patterns during embryogenesis is paramount, not only for finding the ideal cell source and the ideal cellular phenotype for cartilage tissue engineering but also for identification of master regulator genes for targeted differentiation of stem cells.

2 Articular Chondrocytes

2.1 Articular Chondrocytes in Their Natural Environment

Articular cartilage forms the bearing surface at the ends of the major long bones in the joints of the skeleton. It is a highly specialized form of hyaline cartilage, which is attached to subchondral bone at its base and with no cover of perichondrium at the articular surface. As in all cartilage, it contains a uniformly differentiated cell type, the chondrocyte, embedded in a matrix which forms more than 95% of the tissue mass (Fig. 2). The matrix is responsible for the mechanical load bearing, which combines tensile strength related to the dense network of fibrillar collagen (principally collagen II), and compressive resilience, which is dependent on the highly hydrated proteoglycan-rich (principally aggrecan) matrix filling the space. It is this

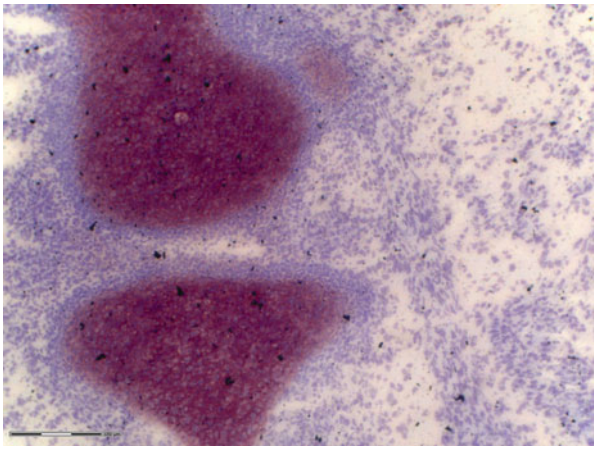


Fig. 2 Interzone formation in mouse hind limbs at E15.5

composite structure of collagen network in a matrix osmotically swollen by negatively charged glycosaminoglycans on the large and small proteoglycans that provides the tissue the ability to withstand repetitive loading in the joint. The chondrocytes maintain the matrix throughout life, and although they proliferate during tissue growth, the cells divide very little during adult life. The major collagen of the cartilage matrix is very long-lived and is estimated to show very little turnover in adult life (Heinemeier et al. 2016). Much of the collagen network we lay down in articular cartilage as teenagers is thus still with us when we die! In contrast the non-fibrillar matrix components are much more dynamically maintained. The prime function of the resident articular chondrocytes is to uphold the balance between maintaining long-lived structural components and constantly replenishing other matrix macromolecules. The viability of chondrocytes is necessary for tissue health, but over many decades the risks to their function accumulate. With aging the number of viable cells in articular cartilage declines, reflecting cell death. There is also potential loss of function due to decline in the protective function of autophagy, accumulation of damaged cellular proteins, endoplasmic reticulum stress, mitochondrial dysfunction, oxidative stress, loss of circadian regulation, and cellular deoxyribonucleic acid (DNA) damage (Lotz and Loeser 2012). These processes may compromise the ability of the chondrocyte to maintain the healthy function of cartilage, either directly or via increased production of inflammatory cytokines and matrix-degrading enzymes. This deregulated function may contribute to joint pathologies, such as osteoarthritis, which is a polygenic and multifactorial disease characterized by cartilage damage as a hallmark, but also changes to all joint tissues and culminating in the mechanical failure and eventual loss of articular cartilage at sites of major load.

2.2 Articular Cartilage Development

In the early embryo, cartilage develops at the end of long bones, such as those in the limbs. The limb buds are formed from paraxial and lateral plate mesoderm emerging from the posterior region of the primitive streak at gastrulation (Cheng et al. 2014). In man, they appear around day 26 for the upper limb and day 28 for the lower limb; in mice they appear around embryonic day 11. Following condensation of the mesenchymal cells, cartilage anlage is formed. At each prospective joint site, an interzone is formed to divide the previously uninterrupted cartilaginous skeletal template (Fig. 2) into its future skeletal elements (Holder 1977; Pacifici et al. 2006; Zhang et al. 2011). Interzone cells can be identified by gene expression of growth/differentiation factor 5 (Gdf5), wingless-type MMTV integration site (Wnt) 4, Wnt9a, doublecortin, and versican, while chondrocytes of the bone anlagen turn on *matrilin-1* and stop doublecortin expression (Storm and Kingsley 1996; Francis-West et al. 1999; Später et al. 2006; Hyde et al. 2007; Koyama et al. 2008; Hyde et al. 2008). Removal of the interzone leads to joint ablation and bone fusion, demonstrating its importance for articular morphogenesis (Holder 1977). Several elegant lineage tracing experiments have demonstrated that *Gdf5*-positive,

matrilin-1-negative interzone cells and their progeny constitute a progenitor cell cohort endowed with joint-formation capacity and which gives rise to the majority of joint tissues and structures, including articular cartilage, ligaments, and synovial lining (Pacifici et al. 2005; Hyde et al. 2007; Hyde et al. 2008; Koyama et al. 2008; Decker et al. 2014; Shwartz et al. 2016). Recent evidence suggests that the outer interzone contributes to the growing epiphysis, while the early-specified *Gdf5*-positive articular progenitor cell pool in the intermediate interzone is supplemented during morphogenesis by a continuous influx of cells from the vicinity of the nascent joint, which migrate into the joint, turn on Gdf5 expression, and contribute to joint development (Pacifici et al. 2006; Hyde et al. 2007; Hyde et al. 2008; Koyama et al. 2008; Decker et al. 2014; Jenner et al. 2014; Ray et al. 2015; Decker et al. 2015; Shwartz et al. 2016).

In cartilage development, the master transcription factor SRY-related HMG-box (SOX)9 drives chondrogenesis enhanced by SOX5 (L-SOX5) and SOX6. Together they directly regulate the expression of genes encoding matrix molecules and maintain the chondrocyte phenotype. Runt-related transcription factor (RUNX)2, which is important in bone formation, is also expressed in the early stages of chondrocyte condensation, terminal differentiation, and hypertrophy. Due to the complexity of the tissues, which is further complicated by regional differences between and within different joints, many extracellular ligands, including transforming growth factor β (TGF-β) family members bone morphogenetic proteins (BMPs), especially BMP2 and BMP4, fibroblast growth factors (FGFs), Indian hedgehog, Wnts acting via β-catenin signaling, as well as inflammatory processes and cell surface/matrix macromolecule interactions, contribute to the regulation of skeletal morphogenesis and joint formation (DeLise et al. 2000; Lefebvre and Smits 2005; Pacifici et al. 2006; Koyama et al. 2008; Kahn et al. 2009; Mundy et al. 2011; Pazin et al. 2012; Jenner et al. 2014; Decker et al. 2014; Cheng et al. 2014; Cleary et al. 2015). The composition of the articular cartilage matrix varies from the superficial to the deep zone, resulting in graded mechanical properties, and this is reflected in the different gene expression pattern of chondrocytes from different zones (Darling et al. 2004). The formation and maintenance of articular cartilage is thus controlled by the cooperative activity of a vast network of signaling mechanisms, which include key transcription factors, extracellular signaling molecules, and cell interactions with matrix and governed overall by biomechanical loading.

2.3 Isolation of Articular Chondrocytes

Chondrocytes from human and animal articular cartilage can be isolated from the tissue by enzymatic digestion or outgrowth from explants. As articular cartilage is both avascular and aneural, it is easy to dissect it free from other tissue without contamination with other cell types. Because of its largely glycolytic metabolism and low cellular oxygen demand, it is also easy to isolate viable cells from refrigerated tissue, and there are studies that indicate that storage can be for several days (Schachar et al. 1994). Protocols for the isolation of chondrocytes from human

articular cartilage are available (Tew et al. 2008; Otero et al. 2012a). In general, the isolation consists of the following steps:

1. Articular cartilage is cut from the subchondral bone and finely dice to improve the efficiency of the subsequent enzymatic digestion.
2. The methods for digestion vary between laboratories. Overnight digestion in 0.08% bacterial collagenase type II in DMEM with 5% or 10% fetal bovine serum (FBS) is satisfactory. Some groups use treatments, such as predigestion (1 h) with trypsin, hyaluronidase, or pronase in order to allow better access of the collagenase to the collagen fibrillar network.
3. Once digested and tissue clumps remain, the cell suspension is passed through cell strainers (100 um pore size) and centrifuged (600 g for 5 min) to obtain a cell pellet. This pellet is gently washed twice in DMEM containing 10% FBS with centrifugation and the cells counted.

Tissue digestion with bacterial collagenase is a harsh process, and it is important not to overdigest. Pretesting batches of collagenase for activity to optimize the concentration and time needed to release chondrocytes without causing cell death can improve cell recovery.

2.4 Expansion

For standard monolayer cultures, the cells are plated on tissue culture plastic at 20,000 cells/cm2. Densities (down to 7500 cells/cm2) can be used and will give more expansion per passage. Where retention of phenotype is important in short-term cultures, higher densities may be desirable to minimize cell spreading and suppress cell division.

Chondrocytes typically grow to confluence in 10 days (note there is some variation due to species and age of the donor) in DMEM containing 10% FBS with medium changes every 2 days once the cells have attached. They can then be split following trypsinization to increase cell number. It is important to understand that from the moment the chondrocytes attach to tissue culture plastic, spread out, and divide, they change their gene expression, and over several passages in monolayer they lose their chondrogenic phenotype (Cheng et al. 2014) (Fig. 3). This dedifferentiation is associated with the loss of rounded shape, dramatic reduction in extracellular matrix synthesis, loss of expression of chondrocyte marker genes (e.g., SOX9 and COL2A1), and increased expression of fibroblastic genes (e.g., COL1A1). Gene expression of COL2A1, which is one of the most sensitive markers of chondrocyte phenotype, rapidly decreases to a low level at passage 3 or 4, while SOX9 gradually declines during six or seven passages. At the same time COL1A1 increases several orders of magnitude in just two passages of culture.

Although clear donor differences exist, re-expression of chondrocyte phenotype is generally possible with human chondrocytes up to passage 4 expanded at a 1:2 split ratio (meaning 16 doublings). However beyond passage 6 the cells lose the

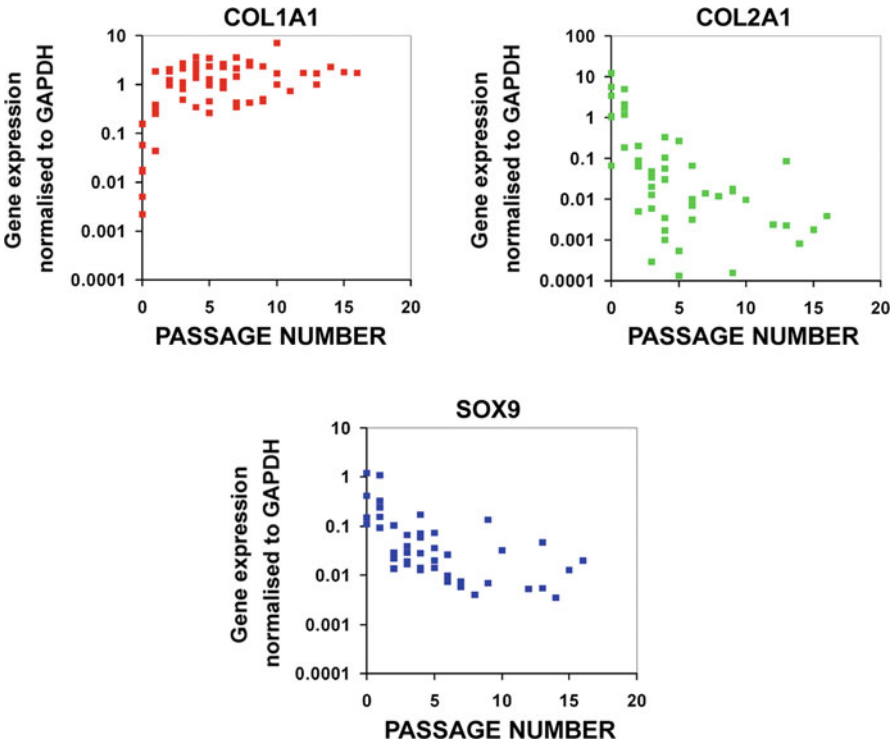


Fig. 3 Gene expression of *COL1a1*, *COL2A1*, and *SOX9* of human articular chondrocytes during expansion (Tew et al. 2008; reproduced with permission from Elsevier, License number 4035361263931)

ability to recover a cartilage extracellular matrix-forming capability, clearly reflecting a more profound change in phenotype. The addition of growth factors platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)-β1, and FGF2 to the culture medium of human chondrocytes is reported to increase the rate of cell proliferation and may also improve the recovery of phenotype in permissive chondrogenic culture conditions (Barbero et al. 2003).

2.5 Chondrogenic Differentiation

2.5.1 Growth Factors

Many studies have reported variable results in the treatment of mature articular chondrocytes with growth factors to stimulate matrix synthesis. This may relate to species differences, tissue sources, passage in culture, and the different chondrogenic conditions under which tests are reported. However, there are some general patterns of response. TGF-β family members (TGF-β1, 2, 3) are able to stimulate matrix synthesis in articular chondrocytes during redifferentiation promoting collagen and

GAG synthesis (Nishida et al. 2000). Insulin-like growth factor-1 (IGF-1) causes upregulation of cartilage extracellular matrix production by mature chondrocytes during organ culture and cartilage repair in vivo (Sah et al. 1994). In contrast in monolayer culture of human articular chondrocytes, IGF-1 had little effect on aggrecan and collagen II gene expression, but was able to increase the expression of these genes synergistically when combined with TGF-β1. FGF2 is a potent mitogen in many cells and at low concentrations can act as an enhancer of collagen and GAG synthesis in cartilage explant cultures (Sah et al. 1994). Interestingly, at higher concentrations there is an inhibitory effect on matrix synthesis, possibly because synthetic activity decreases with increased proliferative activity. Culturing rabbit chondrocytes in monolayer with FGF2 increases the rate of proliferation, stimulates matrix production when cells reach confluence, and makes them more receptive to re-expression of chondrogenic phenotype in three-dimensional culture. However, human articular chondrocytes do not respond to FGF2 alone during monolayer expansion, but the addition of TGF-β1 and PDGF-BB increases proliferation and retention of phenotype as noted above (Barbero et al. 2003).

2.5.2 Gene Transduction

Next to the addition of growth factors, advances in methods of gene transduction have provided new routes to promote cartilage matrix formation by articular chondrocytes. With non-replicating adenoviral, retroviral, and lentiviral vectors, even primary cells can be efficiently transduced. In chondrocytes, adenoviral vectors provide transduction efficiencies of up to 95%, which contrasts with the low efficiencies using nonviral lipid-based methods or electroporation. Adenoviral transduction leads to transient expression of genes for up to 1 to 2 weeks, which is adequate for some purposes. Classic adenoviral vectors are hardly used any more. A main issue is that adenoviral vectors express episomally without integration into the cell nuclear DNA, so when cells divide the vector is diluted not copied. So in the example above at passage 4 of cells transduced passage 0, there would be only 6.25% of the original expression remaining per cell. For more sustained expression, retroviruses and lentiviruses incorporate themselves into the host cell genome and give high transduction efficiency and increased matrix formation, as has been reported when SOX9, TGF-β1, IGF-1, or BMP7 were transduced into chondrocytes (for comparison of different transfection methods: Li et al. 2004; for review on gene therapy for cartilage repair: Madry et al. 2011).

Retroviral transduction of primary human articular chondrocytes isolated from OA cartilage with the transcription factor SOX9 (Tew et al. 2005) promotes retention of chondrocyte-specific responses in passaged cultures, and boosting SOX9 expression made them more sensitive to other cartilage matrix-forming stimuli, including being in 3D culture and responding to growth factors (IGF-1 and TGF-β1). However, the regulation of SOX9 gene expression at the molecular level is not yet well understood. SOX9 expression in chondrocytes is altered by changes in cell shape and actin cytoskeleton integrity, and this may contribute to the re-expression of the matrix-forming phenotype on transfer of cells to 3D culture. During chondrogenic differentiation SOX9 can also be upregulated by BMP2 in mesenchymal-derived

mouse cell lines by sonic hedgehog in chick presomitic mesoderm and by dexamethasone in newborn murine costal chondrocytes.

2.5.3 Physicochemical Environmental Factors

There are several parameters that appear important for the in vitro generation of cartilage tissue with chondrocytes. Some of the changes in gene expression due to monolayer expansion are reversible if cells are placed in 3D culture in cell pellets, in alginate beads, or in suspension on poly-2-hydroxyethyl methacrylate (HEMA)-coated dishes (Tew et al. 2008). This re-expression of phenotype can also be promoted by preventing actin stress fiber development with cytochalasin (Benya et al. 1988) or by inhibition of RhoA effector kinases 1 and 2 (Tew et al. 2009). While it is clear that three-dimensional (3D) culture is needed to support chondrogenic matrix production, it is important that the embedded cells exhibit a rounded cell morphology without developing focal cell attachments and actin stress fibers. This is achieved in scaffold-free culture and in various forms of hydrogels that lack any dominant fibrillar structure. Where fibrillar scaffolds have been tested, those with nanofibers with diameter much smaller than chondrocytes are more successful as they don't encourage cell attachment, whereas larger macrofibers allow chondrocytes to attach and spread as in monolayer (Nuernberger et al. 2011). Thus, the 3D geometry of the construct is irrelevant if the chondrocytes within it are not supported in a chondrogenic morphology.

A further key feature of in vitro systems is maintenance of a very efficient supply of nutrients (Cigan et al. 2016). To achieve this many forms of bioreactor have been tested where nutrient media is perfused through channels in tissue constructs. This can be enhanced by various configurations of mechanical loading, including tensile strain, compressive loading, and hydrostatic pressure (Guilak et al. 2014; Bleuel et al. 2015; Nazempour et al. 2016). Besides stimulating nutrient supply, the mechanical signals themselves are important for normal cartilage development in the embryo (Nowlan et al. 2010). All benefits of loading require cyclical application to be effective, typically at around 1 Hz frequency, resulting in positive effects above a low threshold, but with adverse effects at very high strain or load (Bleuel et al. 2015).

Osmotic stress has been shown to control SOX9 mRNA stability (Tew et al. 2009). These results have provided evidence for a basic chondrocyte "osmosensing" mechanism with a physiological role in regulating cartilage ECM gene expression.

While generating cartilage in vitro may provide well-controlled experimental systems for the investigation of the mechanisms governing matrix formation, it remains unclear if growing tissue in vitro with cells is the preferred option for clinical use (Bernhard and Vunjak-Novakovic 2016). The time needed for prolonged culture and the general immaturity and mechanical weakness of the tissue formed is a challenge, and it is more costly than the use of acellular biomaterial constructs. However, the potential long-term advantage in clinical outcome that a cell-based repair provides may justify this more complex solution.

2.6 Clinical Studies

Joint degeneration in osteoarthritis, including the loss of cartilage at the articulating surface, leads to pain and immobility, which is treated by pain relief and eventually by replacement of the joint with an implant. While the outcomes of joint arthroplasty are good, the incidence of prosthesis failure over time limits the usefulness of this procedure for individuals below the age of 50. Therefore for younger people a desirable treatment for cartilage damage would be the resurfacing of the joint with a functional replacement tissue. Initially, research has focused on the repair of acute localized defects in articular cartilage that frequently occur as a result of sports injury. While the incidence of patients needing such treatment is significantly less than of patients with arthritis, the repair of localized defects has presented an achievable first step in articular surface regeneration.

Cell-based strategies for cartilage repair vary but have a number of common features. For clinical autologous cartilage repair (ACI), articular cartilage derived from the patient is the primary source of cells. The ACI technique consists of a two-stage procedure with first a harvest of a cartilage biopsy that is sent for cell culture expansion, followed by a second-stage operation that includes the cell implantation. A full-thickness cartilage biopsy (2–300 mg of cartilage) is harvested from a low weight bearing area of the knee trans-arthroscopically. The biopsy is put into a tube with biopsy medium, packed, and sent to a high-quality Good Manufacturing Practice (GMP) laboratory for further processing together with 15x9 ml autologous blood collected from the patient made finally to serum. Chondrocytes are isolated from the cartilage by mechanical mincing followed by enzymatic digestion with collagenase and followed by cell seeding into culture flasks. Around 2000–2200 cells/mg of cartilage are isolated. After a total culture time of 14–21 days, the implantation of the cells can be performed with a treatment dose of ca 1–2 million cells/cm2 defects or 30 million cells/ml (Concaro and Concaro 2016). The isolated cells are delivered into the defect site and retained there by some artificial matrix or covering.

The first clinical use of autologous chondrocytes was done in October 1987 (Brittberg et al. 1994). Since that pioneering operation, more than 50,000 implants worldwide of first to fourth generation of ACI have been used. In the first generation, a patient's own periosteal flap was used to cover the defect, and chondrocytes were injected underneath; in the second generation, the periosteum was replaced by a collagen membrane. The third-generation products include culture-expanded chondrocytes seeded onto or into a scaffold that functions as a 3D carrier system for the cells to grow and produce cartilage-specific proteins. The culture period is about 14 days in the scaffold which results in a total culture time of around 5 weeks including the expansion phase for the chondrocytes. Fourth-generation ACI includes a mixture of a direct isolation of chondrocytes and MSCs mixed and seeded on scaffolding or carrier material in a minimally invasive surgical procedure.

The companies that are producing cells for human implantation have adopted different techniques for the cell quality evaluation. The morphology of the cells can

be controlled easily. The sterility of cell culture media is controlled with an alert system 24 hours before implantation, while the viability of cells in suspension is controlled with the use of trypan blue. For bacterial decontamination limulus amebocyte lysate assay could be used (Concaro and Concaro 2016).

To see that the number of non-chondrocytes is low, a special batch of cells are tested for the mRNA expression of synoviocyte-specific genes and identity test for "real" chondrocytes that include mRNA markers of chondrogenic lineage like Sox9. A potency test must identify cells that have the potential to form hyaline-like cartilage. One company has examined expression of the critical components of hyaline cartilage during the chondrocyte manufacturing process and in redifferentiation assays. From these studies "Hyaline1" was identified as a candidate potency marker. Using an assay measuring the ratio of Cart1:Synov1, a large population study of chondrocyte and synovial fibroblast cultures examined the assay's suitability for identity classification (Rapko et al. 2010). Their data suggest that Hyaline1 is predictive of the chondrogenic potential of cells used to manufacture chondrocyte-seeded implants. The viability and quality of directly isolated and seeded cells are more difficult to evaluate as well as the cell attachment percentage post direct cell seeding onto a scaffold implanted into a defect (Rapko et al. 2010).

Long-term results of up to 20 years have been presented for first-generation ACI (Peterson et al. 2010; Niemeyer et al. 2014). Such implants seem to achieve satisfactory clinical results in terms of patient satisfaction, reduction of pain, and improvement of knee function. However, full restoration of knee function cannot be achieved (Horas et al. 2003).

ACI is the cartilage repair method that has been most thoroughly studied in randomized clinical trials (RCTs). In 2016, there exist 17 different RCTs, in which ACI is compared either with other techniques or with different generations of ACI (Horas et al. 2003; Schneider and Andereya 2003; Bentley et al. 2003; Visna et al. 2004; Bartlett et al. 2005; Dozin et al. 2005; Gooding et al. 2006; Knutsen et al. 2007; Zeifang et al. 2010; Basad et al. 2010; Engen et al. 2010; Vanlauwe et al. 2011; Cole et al. 2011; Spalding et al. 2011; Lim et al. 2012; Crawford et al. 2012; Saris et al. 2014; Akgun et al. 2015). In 13 of those studies, ACI was compared with another cartilage repair method (Horas et al. 2003; Bentley et al. 2003; Visna et al. 2004; Dozin et al. 2005; Knutsen et al. 2007; Basad et al. 2010; Vanlauwe et al. 2011; Cole et al. 2011; Spalding et al. 2011; Lim et al. 2012; Crawford et al. 2012; Saris et al. 2014; Akgun et al. 2015), and in 8 of those 13 studies, ACI was compared with the microfracture technique (Knutsen et al. 2007; Basad et al. 2010; Vanlauwe et al. 2011; Cole et al. 2011; Spalding et al. 2011; Lim et al. 2012; Crawford et al. 2012; Saris et al. 2014). In 6 of those 8 studies (Basad et al. 2010; Vanlauwe et al. 2011; Cole et al. 2011; Spalding et al. 2011, Crawford et al. 2012; Saris et al. 2014), the clinical ACI results were significantly better in different parameters than the microfracture results. In the latest published randomized study (Saris et al. 2014), the ACI results were more clinically significant than microfracture at 2 years follow-up, but no difference was noted between the two techniques regarding the repair tissue appearances by magnetic resonance imaging (MRI) or histology.

Several randomized studies on cartilage repair have been reported. However, the variation in type of injury, the extent of injury, and the number of repair methods

used make the results presented in those studies difficult to interpret. These difficulties have been clearly highlighted by Engen et al. (Engen et al. 2010), who disputed if the patients included in the different studies using very strict inclusion criteria are representative of a common population with cartilage injuries. In the future, we will focus much more on the data collected in large registries such as the existing registries used in the Scandinavian countries and similar registries for hip and knee prostheses.

The use of chondrocytes for clinical cartilage repair is expensive. However, a recent British economic analysis estimated that chondrocyte implantation may provide substantial patient benefits over a lifetime horizon, with considerably increased quality-adjusted life years (QALYs) (Elvidge et al. 2016). Despite the increase in costs, the procedure was cost-effective at standard thresholds used in the United Kingdom.

ACI is mainly a two-stage procedure, but one-stage fourth-generation ACI techniques are becoming popular (Cole et al. 2011; Spalding et al. 2011). Direct isolation of a low number of chondrocytes mixed with a larger number of autologous or allogeneic bone marrow mesenchymal stem cells resulted in the induction of proliferation to increase the number of chondrogenic cells needed for the cartilage formation (de Windt et al. 2016). The future of cartilage repair lies in the development of different methods that direct the chondrogenic cells to produce a reliable long-lasting and functional repair tissue. To conclude, ACI is a well-established clinical method, which, however, needs to be improved to perfect the repair of cartilage injuries.

3 Nasal Chondrocytes for Cartilage Tissue Engineering

3.1 Nasal Chondrocytes (NCs) in Their Natural Environment

At birth the nasal septum, except for the vomer "anlage," is completely cartilaginous. In the second half of the first year of life, the septum progressively ossifies in the posterior-anterior direction by a process of endochondral ossification (Schultz-Coulon and Eckermeier 1976). The remaining cartilaginous part (characterized as hyaline cartilage) of the human nasal septum has a specific three-dimensional organization with regard to local differences in cell size and the amounts of extracellular matrix. Chondrocytes, the only cells in septal cartilage, differ in size and shape. They are numerous, small, and flat and they are oriented to the surface of the cartilage in the outer regions. In the intermediate and central zones, they become rarer and have acquired a round spheroid shape with the axis or their orientation more perpendicular to the cartilage surface (Popko et al. 2007) (Fig. 3). The septal cartilage matrix is mainly composed of type II collagen (90–95%, according to its hyaline nature), with small amounts of type IX and XI collagen incorporated in the collagen fibrils (Holden et al. 2008).

Nasal cartilage is tightly connected to perichondrium, consisting of several layers of connective tissue fibers (mainly based on type I collagen) running parallel to the cartilage (Aksoy et al. 2012) (Fig. 4).

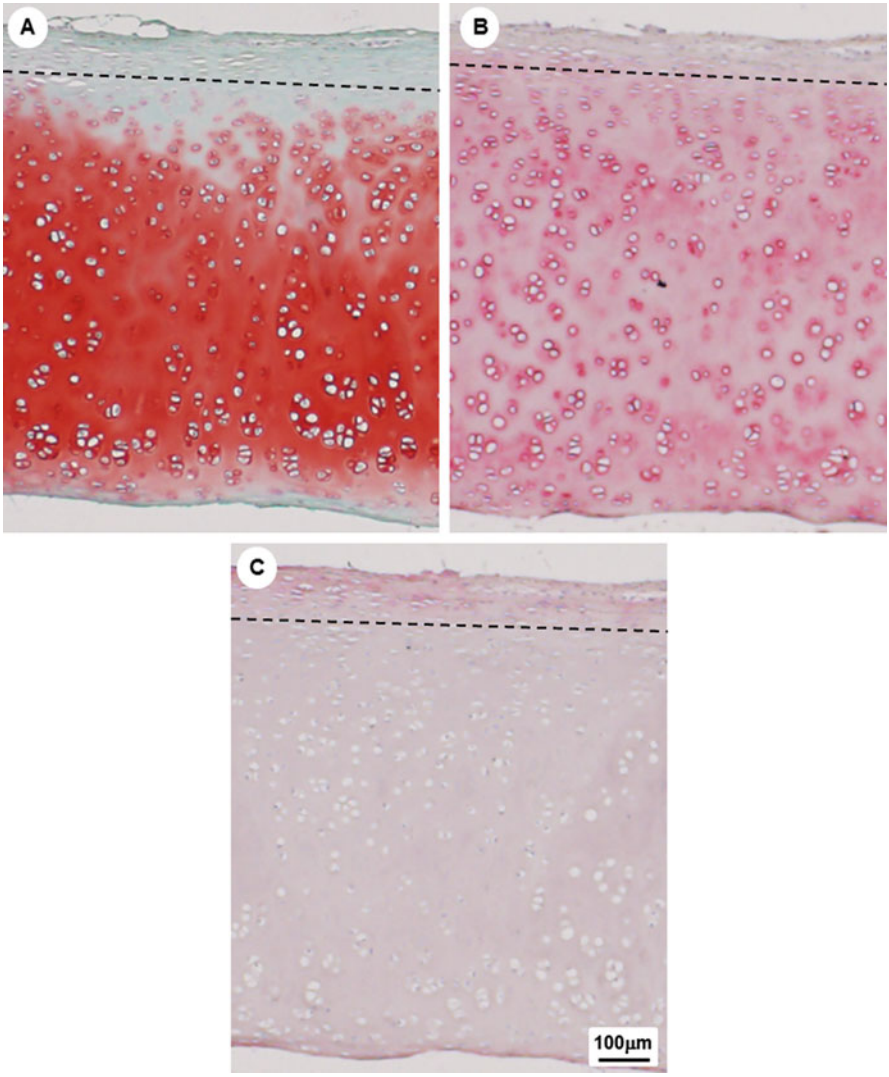


Fig. 4 Histological and histochemical appearance of a nasal septum cartilage biopsy: A Safranin O, B type II collagen, and C type I collagen staining. Dotted lines indicate the interface between cartilage and perichondrium.

3.2 Isolation

Harvesting of nasal cartilage is minimally invasive, can be performed in an outpatient procedure under local anesthesia (Fig. 5a–c), and is associated with less morbidity than the collection of biopsies from other cartilage tissues (i.e., auricular, articular, and costal) (Siegel et al. 2000). NCs are isolated from the cartilage biopsies

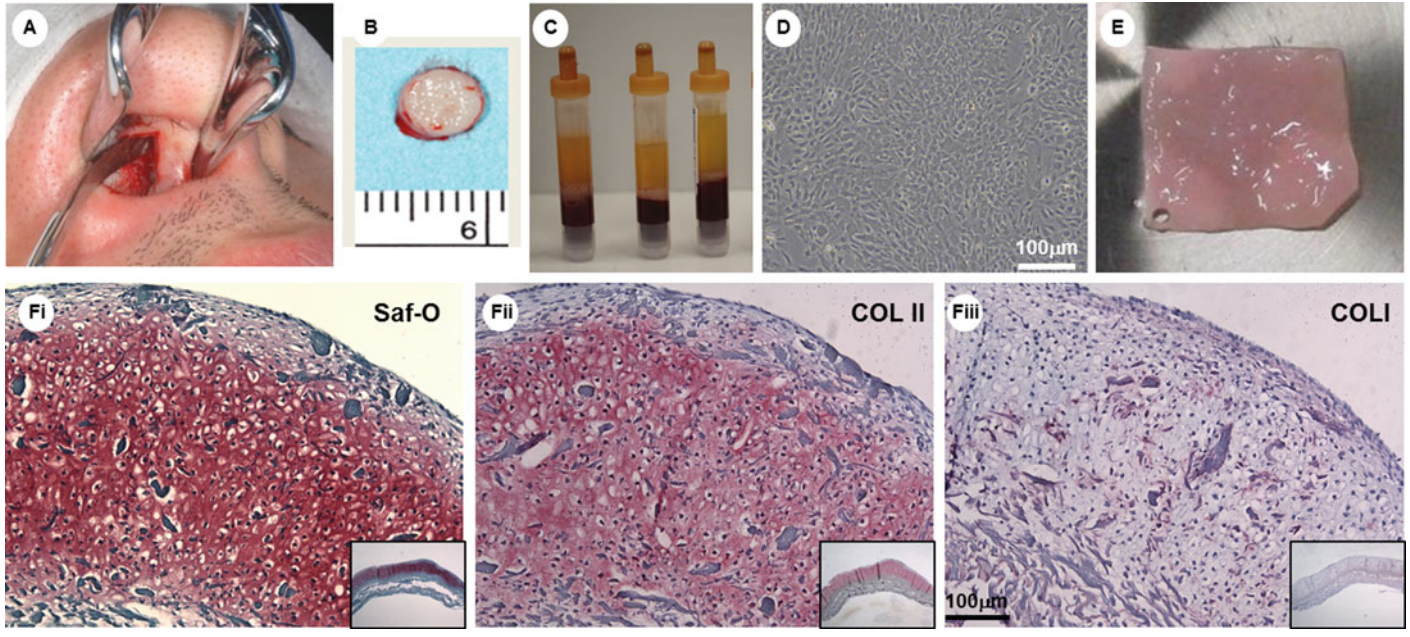


Fig. 5 Example of the manufacturing of the tissue-engineered graft for clinical application (ClinicalTrials.gov NCT 01242618, Swissmedic TpP-I-2010-002) (Fulco et al. 2014). (**a**) Collection of a nasal cartilage biopsy from a patient, this procedure is performed under local anesthesia and results in minimal donor site morbidity. (**b**) Biopsy of nasal cartilage septum and (**c**) blood harvested from the patient after centrifugation (to obtain the serum). (**d**) Expansion of the nasal chondrocytes (NC) using autologous serum. 80–100 million of cells can be generated in 14 days in medium containing TGF-b1 and FGF2). (**e**, **f**) Tissue-engineered cartilage graft generated culturing NC onto type I/III membrane (Chondro-Gide®, Geistlich Pharma AG, Wolhusen/Switzerland, 3x4 cm) in medium containing autologous serum, in the absence of TGF-β: macroscopic (**e**) and histological and immunohistological appearance (Safranin O, type II collagen, and type I collagen staining) (**f-i–f-iii**; the insets are low magnification images of the corresponding grafts). Cartilage graft has translucent appearance and histological characteristics of hyaline cartilage

using collagenase type I, II, or IV (at different combination and concentration, varying from 0.15% to 0.6%) for 12–16 h alone or after an initial short preincubation phase (30–60 min) with pronase (0.2–1%) (Homicz et al. 2002; Tay et al. 2004; Chua et al. 2005; Vinatier et al. 2007; Shafiee et al. 2011; Liese et al. 2013; Elsaesser et al. 2016). Cell yields after enzymatic digestion of the nasal cartilage are estimated to be 2100–3700 cells/mg of tissue (Tay et al. 2004; Chua et al. 2005).

Alternatively nasal chondrocytes can be isolated by outgrowth culture of nasal cartilage fragments (Elsaesser et al. 2016). These cells, due to their higher migratory properties as compared to enzymatically isolated NCs and their surface marker profile more resembling that of mesenchymal progenitor cells (in particular cluster of differentiation (CD)29), are referred to as chondroprogenitors (Elsaesser et al. 2016). However, it is not clear whether this cell population is truly located within the nasal cartilage tissue or derived from the perichondrium, considering that this layer is tightly connected to the septal cartilage and can barely be fully detached from the septum cartilage during the sample harvesting (van Osch et al. 2001a; do Amaral et al. 2012).

3.3 Expansion

NCs exhibit a high proliferation capacity (Fig. 5d) even if derived from the nasal septum of aged individuals (Rotter et al. 2002; Wolf et al. 2008). The proliferation rate of NC improved in the presence of specific growth factors like TGF-β and FGF2 (Bujia et al. 1994; Tay et al. 2004) or culture supplements like insulin-transferrin-selenium (Chua et al. 2005). Importantly Wolf et al. (2008) demonstrated that NCs cultured in medium containing autologous serum, even at low percentage (i.e., 2%), exhibit proliferation rates similar to NCs cultured with medium supplemented with FBS (Wolf et al. 2008). As reported by Tay et al. (2004), taking into account both cell yields and proliferation rate, a nasal cartilage biopsy (50–100 mg) (Fig. 5b) reproducibly yields tens of millions of expanded NCs at passage 2, i.e., sufficient for the generation of autologous grafts of clinically relevant size (Tay et al. 2004) (Fig. 5e).

3.4 Chondrogenic Differentiation

NCs, similar to chondrocytes from hyaline cartilage tissues in other locations in the human body, undergo a process of cell dedifferentiation during monolayer culture, characterized by a gradual acquisition of a fibroblastic morphology and the expression of proteins associated with an undifferentiated mesenchymal cell phenotype (e.g., type I collagen and versican) to the detriment of hyaline cartilage-specific markers (e.g., type II collagen and aggrecan) (Homicz et al. 2002). Expanded/dedifferentiated NCs, however, exhibit excellent capacity to redifferentiate when transferred into an environment supporting a spherical morphology (Fig. 5f). Abundant production of cartilage-specific matrix has been reported for expanded NCs once induced to redifferentiate in micromass pellets (Tay et al. 2004; Wolf et al.

2008; Shafiee et al. 2011; Penuela et al. 2014), in alginate beads (van Osch et al. 2001a; Homicz et al. 2003; Alexander et al. 2006; Chang et al. 2012), in hydrogels (Chua et al. 2005; Vinatier et al. 2007), or in scaffolds based on polyglycolic acid (Naumann et al. 1998; Homicz et al. 2002; Homicz et al. 2003), polyethylene glycol terephthalate/polybutylene terephthalate (Malda et al. 2003), collagen (Scotti et al. 2012), or hyaluronic acid (Farhadi et al. 2006; Candrian et al. 2008; Wolf et al. 2008). Supplementation with specific growth factors during the chondrogenic culture of NCs enhances the accumulation of glycosaminoglycans and type II collagen as well as the biomechanical properties of the generated constructs (Alexander et al. 2010; van Osch et al. 2001a). In particular TGF-β enhances the synthesis of GAG and type II collagen by human NCs cultured in soft agar (Bujia et al. 1996). The supplementation with IGF-1 and growth differentiation factor 5 (GDF5) during the culture of NC in alginate beads results in increased accumulation of cartilage-specific proteins and improved biochemical properties as compared with constructs generated without the growth factors (Alexander et al. 2010).

However, it was also reported that good cartilage matrix deposition by NCs can be achieved in absence of chondrogenic factors in the differentiation medium (Malda et al. 2003; do Amaral et al. 2012; Fulco et al. 2014; Barandun et al. 2015). As shown for the expansion phase, autologous serum can be used in replacement of the fetal bovine serum during the creation of NC-based neocartilage constructs (Wolf et al. 2008; Alexander et al. 2010; Fulco et al. 2014) (Fig. 5e, f). Importantly the cartilage-forming capacity of NC is higher and more reproducible than that of articular chondrocytes (Scotti et al. 2012; Candrian et al. 2008; Pelttari et al. 2014) with lower donor-related dependency (Rotter et al. 2002; Wolf et al. 2008).

Additionally, recent studies have shown that NCs, but not articular chondrocytes, exhibit feature of self-renewal capacity, being able to form cartilage tissue following serial cloning (Pelttari et al. 2014). The higher and more robust cartilage-forming capacity and superior plasticity of NCs as compared to articular chondrocytes can be due to their different embryological origin (i.e., neural crest and mesoderm, respectively) (Pelttari et al. 2014). In this regard, in fact, cells with neuroectodermal origins display remarkable plasticity during tissue development in embryos (Helms et al. 2005) and more robust tissue repair in adults (Leucht et al. 2008).

Due to their reproducible high cartilage-forming capacity and their availability under minimally invasive conditions, NCs may be used as an alternative cell source for the treatment of articular cartilage defects. In vitro studies, performed to address whether NC-derived grafts are compatible for implantation in a joint, demonstrated that NCs respond similarly to articular chondrocytes to physical forces resembling joint loading, can upregulate molecules typically involved in joint lubrication (Candrian et al. 2008), and recover after exposure to inflammatory factors typically present after joint injury (Scotti et al. 2012). Apart from their utilization in the tissue engineering field, nasal cartilage explants can be used to study cartilage responses to specific inflammatory or mechanical insults. In particular bovine nasal cartilage due to its superior degradative responses to inflammatory cytokines (as compared to articular cartilage) can be used to study specific enzymatic/degradative mechanisms responsible for cartilage breakdown in inflammatory conditions. Correro-Shahgaldian et al. (2014), using bovine nasal

cartilage, demonstrated that plowing causes death of the chondrocytes closer to the surface as well as matrix metalloproteinase (MMP)-3 mediated GAG loss (Correro-Shahgaldian et al. 2014).

3.5 Preclinical Animal Studies

Different preclinical investigations in experimental animals have been undertaken to provide proof-of-principle of the clinical potential of NC-based constructs for cartilage reconstructions. In particular, the maturation of human NC-engineered grafts is usually assessed in a subcutaneous pocket of nude mice, i.e., an environment highly vascularized and permissive but not inductive to chondrogenesis (Dell'Accio et al. 2001; Scott et al. 2012). The extent of cartilage matrix production and mechanical properties of NC-based constructs have been reported to increase in such in vivo models (Naumann et al. 1998; Rotter et al. 2002; Vinatier et al. 2007; Dobratz et al. 2009; Kim et al. 2009; Bichara et al. 2010; Chang et al. 2012). Chang et al. (2012) showed that grafts generated by NC recovered from alginate culture after 60 days acquire histological appearance, collagen type II content, and mechanical strength similar to those characteristics of native cartilage (Chang et al. 2012). Similar results were reported by Dobratz et al. (2009) (Dobratz et al. 2009) and Kim et al. (2009) (Kim et al. 2009) using different alginate-NC implants and by Xu et al. (2015) using a NC-cultured poly(glycolic)/poly-L-lactic acid scaffold shaped to mimic the contour of alar cartilage. Pleumeekers et al. (2014) compared the in vivo cartilage-forming capacity of chondrocytes from nasal septum, ear, and knee and mesenchymal stromal cells from adipose tissues and bone marrow. They observed that NC-based grafts develop into cartilage tissues with the highest mechanical properties. The biomechanical functionality of the generated tissues correlated to superior and more homogeneous deposition of matrix components (Pleumeekers et al. 2014). With the goal of the clinical use of engineered cartilage for nasal reconstruction, Farhadi et al. (2006) investigated whether precultivation of tissue-engineered nasal cartilage grafts of clinically relevant size would increase the suture retention strength (a prerequisite for reliable implantation) and the tensile and bending stiffness at 2 weeks post implantation, which is a prerequisite for safe removal of external fixation (Farhadi et al. 2006). The authors demonstrated that preculture for 2 weeks of NC into Hyaff-11 meshes is necessary to engineer NC grafts with enhanced mechanical properties.

Although these results are very promising, nude mice are not capable of mounting a significant immune response. Therefore, these studies cannot predict the prognosis of engineered septal cartilage implanted in an immunocompetent host. Bermueller et al. (2013) recently developed an orthotopic animal rat model for nasal septum repair in which perforation of septal cartilage (to create the defect) and grafting of the cartilage substitute were carried out in the same procedure (Bermueller et al. 2013). Using this model Elsaesser et al. (2014) showed that decellularized xenogeneic collagen scaffold integrates well into septal cartilage defects without causing a

strong inflammatory reaction and also prevents the development of nasal septum perforation (Elsaesser et al. 2014).

As mentioned in the previous subchapter, NCs can be envisioned as good cell source for the repair of articular cartilage defects. Pelttari et al. (2014) have thus tested the capacity of autologous nasal chondrocytes to repair cartilage defects in the goat. In this study the authors demonstrated that NCs are capable of modifying their biologically positional memory, defined by their Hox gene expression, and of adopting their otherwise constitutive molecular identity to the environment of the implantation site (Pelttari et al. 2014). As a result of such plasticity, NCs could directly contribute to the repair of the articular cartilage defects and result in superior outcome compared to articular chondrocytes (Pelttari et al. 2014; Mumme et al. 2016a).

3.6 Clinical Applications

The possibility of generating cartilage tissues with reproducible high quality from nasal chondrocytes has inspired plastic surgeons to use the materials as autologous grafts for the reconstruction of nasal cartilage defects. Fulco et al. (2014) used tissue-engineered cartilage grafts based on NCs as autologous grafts for the reconstruction of the alar lobule of the nose after skin tumor resection in a first-in-man clinical trial (ClinicalTrials.gov, number NCT01242618) (Fulco et al. 2014) (Fig. 5). Their results demonstrated that the engineered grafts could lead to complete structural, functional, and aesthetic satisfaction, bypassing additional morbidity of native tissue harvest.

Expanded chondrocytes from adult individuals, when induced to chondrodifferentiate for short time in vitro, generate a relatively immature cartilaginous tissue. Therefore, as an alternative approach, the cell graft can be incubated for a certain time in the human body of the patient, in an ectopic place (e.g., lower abdomen). The resulting matured cartilage block can be then harvested and transplanted in the defect area. This "heterotopic growth and transplantation" method adopted by Yanaga et al. (2009) for ear reconstruction (Yanaga et al. 2009) can be considered, in principle, for the treatment of microtia and nasal cartilage reconstruction.

As presented in the above chapters, several studies demonstrated that NCs are compatible with environmental features typical of the injured knee (e.g., in terms of responses to inflammatory molecules, mechanical loading, and genetic molecular signature) (Candrian et al. 2008; Scotti et al. 2012; Pelttari et al. 2014). Thus nasal chondrocytes could be used as an alternative cell source for the repair of articular cartilage defects. At the University Hospital Basel (Switzerland), a phase I clinical trial (ClinicalTrials.gov, number NCT 01605201) has been conducted to test the safety and feasibility of implanting a tissue-engineered cartilage graft based on autologous nasal chondrocytes for the regeneration of knee cartilage. For this study nasal chondrocytes (harvested from the nasal septum of the patients) were expanded for two passages (corresponding to 8–10 population doublings) in medium containing autologous serum, supplemented with TGF-β1 and FGF2). After this

expansion phase (lasting 2 weeks), fifty million of nasal chondrocytes are seeded in a type I/III collagen membrane (3x4 cm), and the resulting constructs were cultured 2 additional weeks in medium containing autologous serum, supplemented with insulin and ascorbic acid (Mumme et al. 2016b). The clinical observations of the first ten patients of this study not only indicate safety and feasibility of the procedure but also, together with MRI data, show promising results for efficacy of the treatment as indicated by significant improvement in clinical scores and regeneration of hyaline repair tissue after 24 months (Mumme et al. 2016b).

4 Auricular Chondrocytes

4.1 Auricular Chondrocytes in Their Natural Environment

The cartilaginous structures of the external auricle are formed from the 1st and 2nd pharyngeal arches during fetal development. Auricular cartilage derives from the mesenchyme of the arches and is surrounded by a fibrous tissue layer or perichondrium (Klockars and Rautio 2009). The chondrocytes in the bulk portions of the ear cartilage are generally round or ovoid and may be organized in a columnar fashion. The cells in the subperichondral layers may be flattened and oriented parallel to the surface layers of perichondrium. The cells within the ear cartilage are embedded in an extracellular matrix comprised primarily of type II collagen, with lesser amounts of collagens type I and III. Unlike hyaline cartilage found on the surface of diarthrodial joints, ear cartilage is an elastic cartilage with elastin fibers dispersed throughout the extracellular matrix. The combination of the perichondrium and the elastin bestows incredible flexibility to the external ear and prevents the underlying cartilage from fracturing under tension (Roy et al. 2004; Xu et al. 2001).

To evaluate native and engineered auricular cartilage, histological sections are stained with hematoxylin and eosin (H&E) for overall morphological evaluation; charged stains such as Safranin O, toluidine blue, or Alcian blue for assessing the glycosaminoglycans in the tissues; and, importantly for ear cartilage, stains specific for elastin in the ECM. Immunohistochemistry is commonly performed using monoclonal antibodies to detect various collagen molecules, specifically type I and II collagens, and other collagen types found in lesser amounts.

Unlike the extensive biomechanical testing that has been performed on articular cartilage and engineered articular cartilage, little testing has been reported on ear cartilage. Whereas the compressive modulus is the most important measure of articular cartilage performance, it is less clear which mechanical parameter is reflective of ear cartilage performance. The external auricle is an external appendage that needs to retain an intricate three-dimensional shape on the side of the cranium. In reconstructive procedures, the replacement material must be able to withstand the extreme forces imposed by the skin envelope and scar that can form around the implant. The tensile modulus may be a key measure in determining the function of ear cartilage. Roy et al. have published one of the few articles on the tensile properties of native swine ear cartilage. This report included testing with and without

the perichondrial layer, showing that the perichondrium plays a key role in ear flexibility and prevention of cartilage fracture (Roy et al. 2004). Using membranes in combination with engineered cartilage simulates the perichondral layer and prevents fracture of the neocartilage (Xu et al. 2001; Xu et al. 2005; Jian-Wei et al. 2005). Another study has reported that ear cartilage and engineered ear cartilage are much stiffer than comparable specimens of articular cartilage (Xu et al. 2004). Thus, defining and testing the mechanical performance of ear cartilage is ripe for future study to perfect engineering strategies for ear regeneration.

4.2 Isolation

One of the major obstacles confronting auricular engineering is obtaining sufficient numbers of chondrogenic cells to generate a cartilaginous framework. Auricular chondrocytes from animals can be used as freshly isolated cells in laboratory experiments since large numbers can be collected from whole animal ears. However, the numbers of cells acquired from biopsies of human ears or small remnants obtained from patients with microtia will require propagation to acquire adequate numbers of cells for generating whole external ear structures.

Common protocols involve chopping or mincing the ear cartilage into small particles (<1–2 mm2) and placing the particulates in collagenase type II. Other protocols involve predigestion in pronase or other enzyme cocktails (Pleumeekers et al. 2014). Digestion generally takes 12–24 h at 37 °C. The digested material is passed through cell strainers (40–100 microns) to remove any undigested portions. The cells are then washed in phosphate-buffered saline and centrifuged several times to remove all extraneous enzymes and minimize dead cells. Cells can be counted using trypan blue and a hemocytometer or using flow cytometry.

4.3 Expansion

Human auricular cartilage has low cellularity, averaging about 9–10 million cells/g of human ear cartilage (van Osch et al. 2004; Tseng et al. 2014). Thus, approximately 3 × 10^5 chondrocytes can be harvested from a 30 mg biopsy, which may be a typical biopsy size in microtia patients. It is estimated that 150–300 million cells will be needed to engineer a full adult-sized human ear (Bichara et al. 2010). Thus to obtain sufficient numbers of chondrocytes necessary for the production of a human-sized ear, the cells must undergo in vitro expansion. Using current methodologies, a 300- to 500-fold increase may be required to achieve this goal (Shieh et al. 2004).

Freshly isolated auricular chondrocytes can be placed in culture flasks at a density of 3–5 × 10^3 cells/cm2 and propagated in standard medium formulations consisting of Dulbecco's Modified Eagle's Medium (DMEM) or Ham's f-12. Common supplements are fetal bovine serum (10%), ascorbic acid, antibiotics/antimycotics, L-glutamine, and nonessential amino acids. Other reported additives

include glucose, sodium pyruvate, insulin-selenium-transferrin (ITS), and growth factors such as FGF, TGF-β, and PDGF (Tay et al. 2004).

There are convincing data that prolonged monolayer culture of auricular chondrocytes also leads to dedifferentiation as is the case in cultured articular and nasal septum chondrocytes, whereby the cells lose their chondrocyte phenotype and have diminished capacity to produce typical cartilage ECM matrix molecules, such as collagen type II, and they become fibroblast-like (Tay et al. 2004; Saadeh et al. 1999). Any resultant engineered tissue from dedifferentiated chondrocytes lacks the histological, biochemical, and biomechanical characteristics of native cartilage (Homicz et al. 2002; Passaretti et al. 2001). Supplementation of culture medium with growth factors has been shown to prevent, or possibly even reverse, dedifferentiation during expansion in monolayer culture (Tay et al. 2004, van Osch et al. 2001b) when they are passaged.

4.4 Chondrogenic Differentiation

Suspension of chondrocytes in three-dimensional matrices or culture systems that mimic their native environment has been shown to facilitate redifferentiation permitting cells to recover their chondrogenic phenotype (Deshmukh and Kline 1976; Nevo et al. 1972; Norby et al. 1977; Horwitz and Dorfman 1970; Miller 1976; Glowacki et al.; 1983; Benya and Shaffer 1982). Growth factors seem to impact positively the in vitro and in vivo growth of chondrocytes and could offer an important contribution to clinical application by both stimulating chondrocyte expansion and enhancing their ability to produce matrix to increase the yield of neocartilage. Similar to articular and nasal septum chondrocytes, FGF2 prevents chondrocyte dedifferentiation, while TGF-β and IGF-1 promote redifferentiation of rabbit and human chondrocytes cultured in vitro (van Osch et al. 2001b). The combination of TGF-β and IGF-1 can stimulate the production of glycosaminoglycans (GAG) and collagen type II when added to serum-free medium. Terada et al. postulated that chondrocytes from different species require different growth factors for improved matrix formation (Terada et al. 2005). Researchers also have shown variability in the redifferentiation response of chondrocytes from several cartilage sources to scaffolds and growth factors (Homicz et al. 2002). In a case series report, Yanaga et al. (2009) utilized FGF2 to expand autologous chondrocytes harvested from patients with microtic auricles (Yanaga et al. 2009). The cells were implanted into a subcutaneous pocket on the fascia of the patient's lower abdomen and allowed to form new cartilage ECM. A block of cartilage was successfully harvested after 6 months and an earlike framework resembling that usually cut from costal cartilage was carved and implanted. Although successful in this circumstance, the ideal combination of growth factors necessary for proliferation and preservation of the chondrogenic phenotype remains unknown. A recent study using human and sheep chondrocytes demonstrated that a chondrocytic phenotype could be maintained by extensively expanded auricular chondrocytes propagated in media supplemented with bFGF (Tseng et al. 2014, Fig. 6). The neocartilage that formed from these

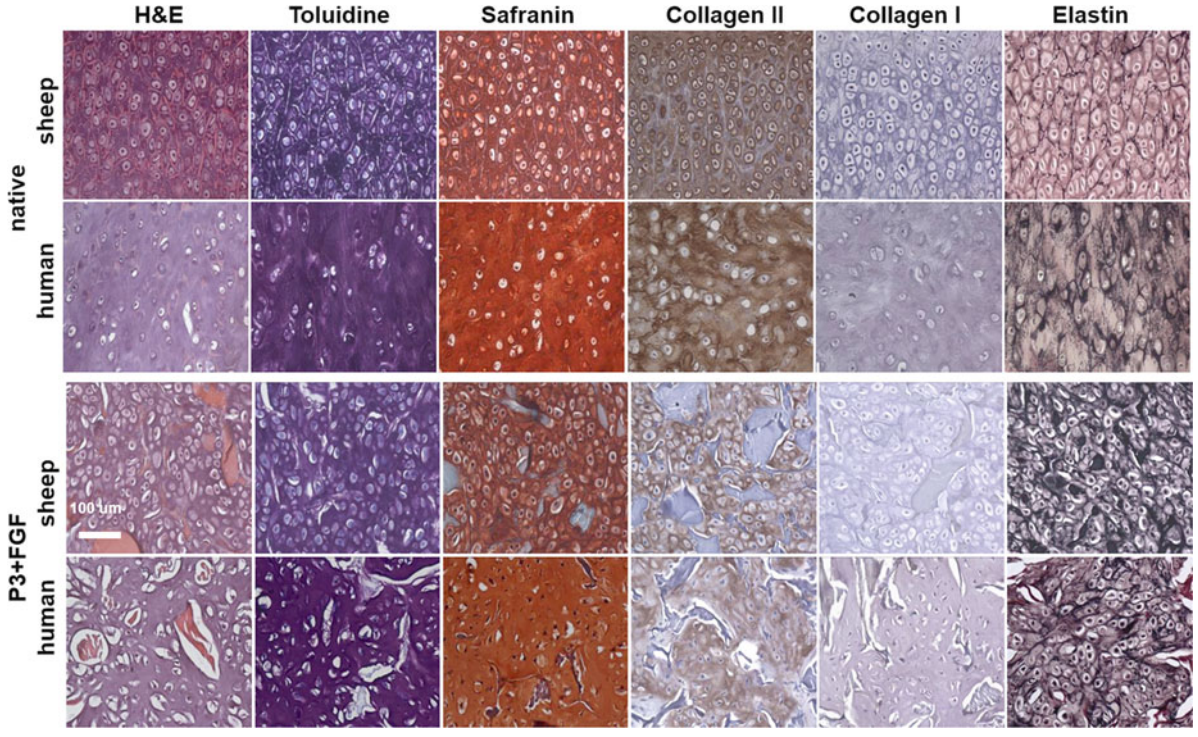


Fig. 6 Histological and biochemical assessment of elastic cartilage engineered from passaged P3 auricular chondrocytes expanded in the presence of bFGF after 6 weeks in nude mice (bottom two rows) and compared to native sheep and human auricular cartilage (top two rows) . Neocartilage formed from both sheep and human cells as evidenced by positive toluidine blue, Safranin, collagen type II, and elastin stains. Elastin fibers were dense and strongly stained. Collagen scaffold fibers stained red on H&E-stained sections. Scale bar: 100 µm (Tseng et al. 2014). M.R. was granted permission for reproduction of the figure from SAGE Publications, license number 4037110283260

cultured cells also contained elastin in the newly formed ECM. Advances in polymer technology will permit scaffold modification to facilitate the controlled release of growth factors concurrent with scaffold degradation (Isogai 2013; Sridhar et al. 2014). Future studies should focus on understanding the influence of growth factors on in vivo neocartilage formation.

4.5 Preclinical Studies

A variety of chondrocyte sources have been investigated in both immunodeficient and immunocompetent animal models for auricular cartilage engineering with varying degrees of success. Reproducible neocartilage formation has been demonstrated successfully in immunodeficient mice or rats using chondrocytes from swine, ovine, bovine, canine, and lapine species (Shieh et al. 2004; Isogai et al. 2005; Kusuhara et al. 2009; Saim et al. 2000; Xu et al. 2004; Zhou et al. 2011). Kusuhara et al. (2009) compared the performance of four bovine chondrocyte sources – articular, auricular, costal, and nasoseptal – in engineered ear-shaped constructs in nude mice (Kusuhara et al. 2009). Cells were seeded onto poly-L-lactic acid (PLLA)/polycaprolactone (PCL) copolymer human ear-shaped scaffolds and implanted for up to 40 weeks.

While constructs seeded with auricular chondrocytes retained an acceptable contour, those seeded with articular chondrocytes decreased in size. Constructs engineered using costal chondrocytes demonstrated a high relative gene expression of bone sialoprotein, and rigid calcified protrusions were also identified, suggesting ossification. Scaffolds seeded with nasoseptal cells were thicker than those seeded with auricular chondrocytes and demonstrated prominent Safranin O staining. In another comparative study, Xu et al. used fibrin gel and swine auricular, costal, and articular chondrocytes to engineer neocartilage in nude mice and demonstrated that auricular cartilage constructs increased in dimensions by 20–30%. Furthermore, auricular constructs had the highest modulus and GAG content (Xu et al. 2004).

In comparison to published studies using animal chondrocyte sources, a limited number of studies using human chondrocytes have been carried out. Human costal and auricular chondrocytes have been investigated with varying degrees of success (Ting et al. 1998; Park et al. 2004). Park et al. found that auricular neocartilage engineered in nude mice using porous polyglycolic acid (PGA)/PLLA scaffolds had mechanical properties similar to native auricular cartilage, but there was irregular distribution of chondrocytes (Park et al. 2004). Studies engineering neocartilage constructs using human cells from microtic (Saadeh et al. 1999; Kamil et al. 2004a) and pediatric (Rodriguez et al. 1999) auricles have been used to generate tissue that resembles cartilage. Most studies have focused on isolation techniques and characterization of the cells and the neocartilage generated (Saadeh et al. 1999; Tay et al. 2004; van Osch et al. 2001b), yet these studies do not demonstrate that human chondrocytes can be used successfully to engineer an auricle of the shape and size of humans. Nonetheless, these studies lay a solid foundation for future work.

4.6 Generating Ear Shapes

Reconstructing or replacing the intricately shaped external auricular cartilaginous structures may require multiple surgical procedures during reconstruction. Ear-shaped frameworks currently used in clinical practice include hand-carved autologous costal cartilage and high-density porous polyethylene, which are associated with various limitations and degrees of success (Lee et al. 2010; Bauer 2009; Hohman et al. 2014). Research from Cao et al. is largely credited with introducing a tissue engineering approach for generating cartilage in the shape of an ear when they reported on the use of bovine articular chondrocytes placed on a PGA scaffold and externally stented in the shape of an ear (Cao et al. 1997). Since then, numerous reports have been published on generating ear-shaped frameworks using various animal cells in immunocompromised rodent models (Xu et al. 2005; Shieh et al. 2004; Zhou et al. 2011). Xu et al. demonstrated that, by incorporating a perichondral layer around swine chondrocytes embedded in fibrin, the ear-shaped constructs on the back of nude rats were highly flexible (Xu et al. 2005).

The generation of ear cartilage and ear shapes in normal immunocompetent animals has been more difficult. Cao et al. found that Pluronic F-127 mixed with autologous auricular chondrocytes causes a minimal inflammatory response compared to either calcium alginate or PGA when implanted subcutaneously in a swine model for a 6-week period (Cao et al. 1998). Despite the ability to generate contiguous neocartilage in the host, clinical application of Pluronic F-127 is limited by its inferior biomechanical properties and the inability to maintain a predetermined shape. In an effort to overcome this limitation, Pluronic F-127 with freshly isolated autologous auricular chondrocytes was injected into a surgically created subcutaneous skinfold channel in the shape of a human auricular helix in a swine model (Saim et al. 2000). However, despite engineering a construct with the size and shape approximate to the human auricular helix, the skin channel failed to resemble a complete auricular-shaped structure. A later study demonstrated that a tissue-engineered human auricle of normal size and anatomic definition can be generated in an immunocompetent large animal model (swine) using a mold technique to incorporate autologous auricular chondrocytes in biodegradable polymers (Kamil et al. 2004b). Recently, Bichara et al. have shown that auricular chondrocytes from sheep placed on a collagen scaffold can produce sustainable ECM in an autologous sheep model by employing a 2-week preculture period during which the cell-scaffold construct is subjected to continuous oscillating motion (Bichara et al. 2014). The strategy also requires substituting autologous serum media in the final phases of preculture to avoid any potential reactions to foreign antigens that may be present in FBS. In a follow-up study, Pomerantseva et al. showed that this methodology could be used successfully to generate ear cartilage of the size and shape of humans in the subcutaneous space of sheep (Pomerantseva et al. 2016; Fig. 7).

Minimal research has been carried out with human chondrocytes and engineered into defined human ear shapes. Ting et al. were the first to combine human costal chondrocytes with fibrin gel to form three-dimensional ear-shaped cartilage in the nude mouse model (Ting et al. 1998). However, over the 8 week in vivo period, constructs

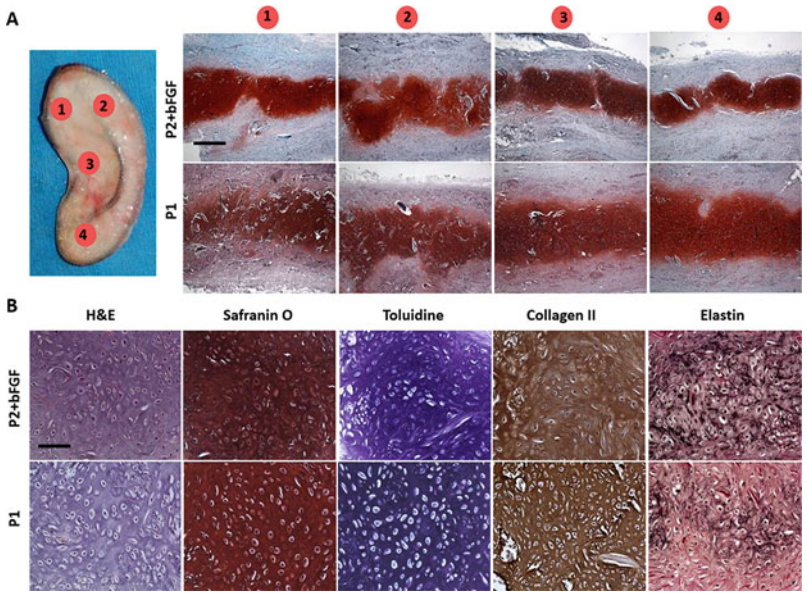


Fig. 7 Neocartilage quality in ear-shaped constructs after 20 weeks implantation. Neocartilage in all four biopsies taken throughout ear scaffold stained intensely with Safranin O (**a**). Neocartilage from P2 + bFGF and from P1 chondrocytes was high quality as confirmed by cartilage-specific stains (**b**). The elastin was more prominent in P2 + bFGF neocartilage. Scale bars: 500 μm (**a**) and 100 μm (**b**) (Pomerantseva et al. 2016; Mary Ann Liebert, Inc. grants authors permission to use their images for books)

undergo severe volume loss and shape deformation, possibly due to the use of abnormal costal cartilage harvested from a patient with Marfan's syndrome and pectus excavatum. Further work by Haisch et al. (2002) investigated the feasibility of engineering an ear-shaped construct from a biopsy of human nasoseptal chondrocytes (Haisch et al. 2002). Following cell expansion, the cells were seeded onto PGLA-PLLA scaffolds and cultured for up to 6 weeks in a bioreactor and implanted in nude mice for 6 and 12 weeks. The implants maintained their original shape and demonstrated only a slight decrease in size. This is an example of a combined in vitro-in vivo approach that permits the preservation of specifically shaped structures. Although there are numerous studies on generating cartilage from human auricular chondrocytes, little has been reported using these cells for generating ear shapes.

5 Meniscus Cells

5.1 Meniscal Chondrocytes in Their Natural Environment

The knee joint contains a medial and a lateral meniscus that is located between the femoral condyle and the tibial plateau. The meniscus has region-specific vascularization and neural innervation; two regions can be distinguished: the outer, vascular/

neural region (red-red zone) and the inner, avascular/aneural region (white-white zone), separated by the red-white region. The healing capacity of each area is directly related to vascularization. Native meniscus contains three distinct cell types: round fibrochondrocytes, which are located in the inner two thirds of the meniscus; elongated fibroblast-like cells, which are mainly found in the outer third; and fusiform superficial zone cells (Nakata et al. 2001; Verdonk et al. 2005; Makris et al. 2011). Fibrochondrocytes are the main meniscal cells (MC) used for tissue engineering (reviewed by Makris et al. 2011).

5.2 Isolation and Expansion

Due to the limited availability of meniscal tissue as source for MCs, donor site morbidity associated with tissue harvesting, and the low MC density in the meniscal tissue, obtaining a sufficient number of MCs for tissue engineering is difficult. For primary MC isolation, meniscal biopsies are harvested using aseptic technique, washed in phosphate-buffered saline, and minced and digested using 3.3 ml collagenase solution (500 U/ml) per 1 g tissue. Depending on the duration of the collagenase digestion period, either enzymatically released cells or meniscus dices, obtained after centrifugation, are washed with MC medium (Dulbeccos's Modified Eagle's Medium (DMEM) high glucose, 10% fetal calf serum, 1 mM sodium pyruvate, 1% penicillin/streptomycin), transferred into a culture flask, and incubated in a humidified atmosphere at 37 °C and 5% CO_2 (Nakata et al. 2001; Cui et al. 2012; Kremer et al. 2017). In meniscus explant cultures, migration of MCs out of the meniscus pieces onto the culture dish surface can be observed within a week of incubation. MC can be characterized by their stretched morphology typical for elongated fibroblast-like chondrocytes and immunohistochemical staining for meniscal markers such as Col I and aggrecan.

5.3 Chondrogenic Differentiation

Analogous to hyaline chondrocytes, fibrochondrogenic native meniscus cells dedifferentiate during monolayer in vitro expansion, losing their typical meniscal and chondrogenic markers and properties, and only redifferentiate when transferred into a 3D environment (Freymann et al. 2012). This is further complicated by the apparent population heterogeneity of MCs, which is reflected by variable performance of MCs in tissue engineering depending on their harvest location. Cells isolated from the inner and outer regions of the meniscus both possess multilineage differentiation capability, have equivalent ECM production and differentiation potential toward the chondrogenic phenotype, and interact synergistically with MSC, enhancing matrix formation. However, MCs from the outer region show greater phenotypic plasticity and are more potent in suppressing hypertrophic differentiation of co-cultured MSCs, whereas MCs harvested from the inner meniscus maintain a more chondrogenic phenotype in monolayer culture (Upton et al. 2006; Mauck et al. 2007; Furumatsu et al. 2011; Croutze et al. 2013).

Although tissue engineering with 100% MC would closest simulate native meniscus, co-culturing MC with MSC may reduce the quantity of MC required to reach a clinically or experimentally relevant number of cells while at the same time facilitating differentiation of MSC toward a fibrochondrogenic phenotype with less hypertrophy and increasing ECM formation (Tan et al. 2010; Cui et al. 2012; Saliken et al. 2012; Hubka et al. 2014; Kremer et al. 2017).

6 Chondrocyte Cell Lines

Since it is difficult to maintain robust cartilage-specific phenotype in monolayer cultures of primary human chondrocytes and they tend to dedifferentiate when they are passaged (Goldring et al. 1986; Aulthouse et al. 1989; Kolettas et al. 1995), researchers have attempted to develop chondrocyte cell lines with variable success. Attempts to immortalize chondrocytes from mouse, rat, and other species with viral oncogenes have generated cell lines with high proliferative capacities and at least some differentiated chondrocyte properties (Gionti et al. 1985; Mallein-Gerin et al. 1995; Thenet et al. 1992). Chondrocyte cell lines have also arisen spontaneously from fetal rat calvaria (Grigoriadis et al. 1988; Bernier and Goltzman 1993) or have been derived from transgenic mice harboring the temperature-sensitive mutant of simian virus 40 (SV40) large T antigen (TAg) (Lefebvre et al. 1995; Mataga et al. 1996). Human chondrosarcoma cell lines express some aspects of the chondrocyte phenotype but are tumorigenic (Block et al. 1991; Takigawa et al. 1991). The generation of cell lines that can undergo chondrogenesis and terminal differentiation to the hypertrophic phenotype has been more successful due to the availability and plasticity of progenitor cell populations. For example, cells derived from the ribs of transgenic mice harboring the temperature-sensitive mutant of simian virus 40 (SV40) large T antigen (TAg) are able to undergo hypertrophic differentiation, and bone marrow-derived mesenchymal stem cells derived from these mice contain osteogenic, adipogenic, myogenic, as well as chondrogenic progenitor cells (Lefebvre et al. 1995; Mataga et al. 1996). Several chondrogenic cell lines derived from mice or rats, including ATDC5, C3H10T1/2, RCJ3.1, CK2, and C1, are now used widely in the field (Johnstone et al. 2003).

6.1 Immortalized Human Chondrocytes

Stable expression of SV40-TAg using plasmid or retroviral vectors has yielded immortalized human chondrocyte cell lines that are useful for studying the regulation of gene expression under defined conditions (Benoit et al. 1995; Steimberg et al. 1999; Goldring et al. 1994, Goldring 2004a, b). Human articular chondrocyte cell lines have also been established using temperature-sensitive SV40-TAg (Robbins et al. 2000), the human papilloma virus type 16 (HPV-16), early function genes E6 and E7 (Grigolo et al. 2002), and telomerase (Piera-Velazquez et al. 2002). Strategies for generation and characterization of human chondrocyte cell lines have been

described previously in detail (Goldring 2004a, b). Approaches for culturing immortalized chondrocytes as reproducible models for evaluating cartilage-specific functions are summarized below.

6.2 Culture of Immortalized Human Chondrocytes

Since prolonged passaging of immortalized cell lines will select for rapidly growing cells, immortalized chondrocytes should be expanded as soon as they are established, frozen down in liquid nitrogen, and retrieved periodically to renew cell stocks for experimental procedures. The application of strategies that maintain high cell density and attenuate cell proliferation in cultures of immortalized chondrocyte cell lines will yield the best results. Established cultures of rapidly growing cell lines are plated at split ratios 1/5 to 1/10 immediately upon reaching confluence, but not more than twice weekly. Clonal expansion usually results in type II collagen-deficient cell lines that express type I and type III collagens in monolayer culture (Oxford et al. 1994; Benoit et al. 1995; Steimberg et al. 1999). One strategy that slows the loss of phenotype is the maintenance of monolayer cultures of immortalized chondrocytes as nonclonal populations. This preserves the potential cellular interactions among clonal cell types that aid in survival and permits maintenance of synthetic properties.

Stable integration of immortalizing genes disrupts normal cell cycle control, but does not preserve the expression of the *COL2A1* or other cartilage-specific genes (Goldring et al. 1994; Robbins et al. 2000; Goldring 2004b; Grigolo et al. 2002). Gene profiling studies, in which the culture medium is supplemented with serum, show high expression of genes associated with the cell cycle and low expression of genes encoding extracellular matrix and matrix-degrading proteinases, even though Sox9 expression is detected (Finger et al. 2003, 2004; Gebauer et al. 2005). One strategy for reversing the loss of phenotype is to select chondrocytes for the ability to survive in suspension by passaging them through a three-dimensional culture system (Goldring et al. 1994, 2004b). These include cells embedded in alginate (Kokenyesi et al. 2000; Robbins et al. 2000) or hyaluronan (Grigolo et al. 2002) or suspended on polyHEMA-coated dishes (Piera-Velazquez et al. 2002). However, immortalized chondrocytes may continue to proliferate in 3D culture, and in systems such as alginate, which cannot be remodeled, necrotic clusters will form as the cells continue dividing after filling the space. Insulin-like growth factor (IGF)-I or insulin added in serum-free medium or as a constituent of serum seems to be a universal basal requirement for enabling chondrocyte phenotype in these culture systems.

Pellet cultures are sufficiently organized to permit deposition of matrix in situ (Olivotto et al. 2008, 2013). They have been used to study terminal differentiation and hypertrophy because they mimic the distribution of cells within the growth plate. Interestingly, long-term pellet culture of a clonal cell line of tsTAg-immortalized human articular chondrocytes at 37 °C, the nonpermissive temperature for proliferation, induced expression of type X collagen, a hypertrophic chondrocyte marker not found in normal articular cartilage (Oyajobi et al. 1998). Arrest of cell proliferation

and activation of type X collagen expression was observed when the serum concentration was reduced from 10% to 2% or lower. Ascorbic acid and treatments such as thyroxine 1,25-dihydroxyvitamin D_3, retinoic acid, or dexamethasone promote terminal differentiation in vitro.

6.3 Application of Immortalized Chondrocytes

Immortalized human chondrocytes have been used to establish micromass cultures as a rapid and reproducible screening assay for chondroprotective molecules, anti-inflammatory drugs, or viral vectors for gene therapy to test their influence on chondrocyte metabolism (Greco et al. 2011). Based on the protocol described by De Bari et al. (2001) using human periosteum-derived cells, the approach involves pipetting 20 μL of cell suspension (at a density of 2.5×10^7 viable cells/ml) into each well of a 24-well plate, allowing cell attachment for 3 h period without added medium, and gently adding growth medium before culturing for a further 24 h, changing to serum-free, insulin-supplemented medium, and proceeding to test the gene expression response profiles of molecules such as IL-1ß, TGF-ß1, and BMP2 (Greco et al. 2011). These micromass cultures have been used to assess the effects of gene overexpression using viral and nonviral expression vectors and the responses to pharmacological agents (Greco et al. 2011, 2014). An advantage is that the micromasses can be stained with Alcian blue and harvested for quantitative measurement of sulphated glycosaminoglycans (Greco et al. 2011).

Success in establishing immortalized human chondrocyte cell lines that can be used as models for studying cartilage breakdown and repair depends not only upon the source and developmental state of the tissue from which the cell lines are established but also more importantly upon the ability of the culture system to support the differentiated phenotype. Immortalized chondrocytes cannot be considered as substitutes for primary cultures (Otero et al. 2012a), but are useful for validation and further elucidation of mechanisms uncovered in non-immortalized chondrocytes. Furthermore, immortalization of osteoarthritic chondrocytes will not necessarily stabilize characteristics that are observed in primary cultures of these cells, unless they are hereditary features of the original chondrocytes in situ. However, immortalized cell lines may be useful as reproducible culture systems for modeling chondrocyte functions. Regardless of the immortalization strategy, however, matrix protein synthesis and secretion in general and chondrocyte-specific phenotype in particular may be reduced after selection and expansion in monolayer culture. Thus, short-term incubation in medium with a very low serum concentration or in serum-free defined medium supplemented with an insulin-containing serum substitute has been used as an experimental strategy (Goldring et al. 1994; Robbins et al. 2000; Grigolo et al. 2002; Kokenyesi et al. 2000).

Immortalized chondrocyte cell lines serve as reproducible models to study the expression of chondrocyte-specific matrix genes and to examine the effects of cytokines and growth/differentiation factors on chondrocyte phenotypic markers. In 3D cultures, it is also useful to characterize the proteoglycans synthesized

(Grigolo et al. 2002; Kokenyesi et al. 2000); stain the matrix components with Alcian blue, toluidine blue, or other dyes; or perform immunohistochemistry using specific antibodies against these proteins. Other features of the chondrocyte phenotype include the expression of the three members of the SOX family of transcription factors, L-Sox5, Sox6, and Sox9 (Osaki et al. 2003), which are required for chondrocyte differentiation during development. The capacity to respond to IL-1b by increasing expression of cyclooxygenase (COX)2 and MMP13 is somewhat dependent upon the differentiated phenotype (Thomas et al. 2002). Integrin profiles should be consistent with those expressed on normal chondrocytes, but also may reflect the types of integrins found on proliferating cells (Loeser et al. 2000). However, immortalized chondrocytes should not be considered as substitutes for primary chondrocytes, which should be used ultimately to validate key findings.

Chondrocyte cell lines have served most beneficially as models to explore the cytokine- and growth factor-induced signaling pathways and transcription factors involved in the regulation of gene expression. Human chondrocytic cell lines derived from various sources have been used to study signaling pathways involving specific protein kinases (Robbins et al. 2000; Thomas et al. 2002; Hamamura et al. 2009), cellular responses such as apoptosis (Healy et al. 2005; Zhu et al. 2010; Toegel et al. 2008), and gene expression profiles in response to regulation by interleukin-1 (IL-1), BMPs, and other regulatory ligands (Finger et al. 2003; Ijiri et al. 2005; Locklin et al. 2001). Because they can be cultured in sufficient numbers and since primary chondrocytes are very difficult to transfect, immortalized chondrocytes are convenient models for examining transient or stable expression of reporter genes driven by promoter and enhancer sequences, such as those regulating transcription of *COL2A1* (Osaki et al. 2003; Peng et al. 2008; Tan et al. 2003), *MMP13* (Hashimoto et al. 2013; Otero et al. 2012b; Surridge et al. 2009; Im et al. 2003), *COX2* (Grall et al. 2005), and/or expression of recombinant wild-type and mutant proteins (examples given: Attur et al. 2000; Thomas et al. 2002; Jayasuriya et al. 2012; Luan et al. 2008). Co-expression of wild-type or dominant-negative mutants of transcription factors, protein kinases, and other regulatory molecules, mediated by plasmid or viral vectors, may be performed to further dissect the mechanisms involved. They have been used to examine specific DNA binding activities in nuclear extracts (Osaki et al. 2003; Peng et al. 2008; Tan et al. 2003) and to test approaches for gene therapy using viral vectors (Ulrich-Vinther et al. 2002) and RNA interference (RNAi) strategies (Zwicky et al. 2002; Conde et al. 2016; Jayasuriya et al. 2012; Nieminen et al. 2010).

Chromatin immunoprecipitation (ChIP) assays are generally limited by the requirement for high cell numbers, the differences in cross-linking efficiencies between low abundance transcription factors and DNA, and the availability of antibodies of high quality and affinity to detect the protein of interest. Chondrocyte cell lines have served as useful tools to optimize and set the conditions required for reproducible and specific ChIP assays, which may be not be feasible using primary chondrocytes (Peng et al. 2008; Otero et al. 2012b; Hashimoto et al. 2013; Grall et al. 2005; Shimada et al. 2016).

Culture of immortalized chondrocytes in 3D scaffolds is a useful approach for experimental tissue engineering applications. Although initially not preferred for

clinical applications, immortalized human mesenchymal stem cells may serve as useful cellular components in tissue engineering studies because the chondroprogenitors may be induced to form hyaline cartilage under defined conditions (Kitagawa et al. 2013; Wu et al. 2011). Many studies have been carried out as proof-of-principle using immortalized chondrocytes from nonhuman sources (Lamplot et al. 2015; Ni et al. 2014; Takazawa et al. 1999; Zamperone et al. 2013). Further work is required to fully exploit the potential of using immortalized chondrocytes in cartilage tissue engineering studies.

7 Mesenchymal Stem Cell-Derived Chondrocytes

7.1 Mesenchymal Stem Cells (MSCs) in Their Natural Environment

Mesenchymal stem cells (MSCs) constitute a population of progenitor cells originally identified by Friedenstein et al. for their capacity to form fibroblast-like colonies in vitro and to give rise to bone when heterotopically transplanted in vivo (Friedenstein et al. 1966; Tavassoli and Crosby 1968; Friedenstein et al. 1970, 1974, 1976). Later, Caplan et al. proposed their multilineage differentiation potential through the description of the mesengenic process, in which a unique mesenchymal progenitor could give rise to multiple tissue phenotypes, including chondrocyte-like cells (Caplan 1991). Only recently, the in vivo natural environment of MSC, known as "the niche," was described as perivascular in multiple adult tissues (Sacchetti et al. 2007; da Silva Meirelles et al. 2008; Caplan 2008; Crisan et al. 2008), as well as during development where skeletal progenitors reside during cartilage and bone formation (Diaz-Flores et al. 1991; Brighton et al. 1992; Maes et al. 2010). Within the niche, MSCs closely interact with other local resident cells, modulating the intrinsic homeostasis of the tissue (e.g., cell proliferation and differentiation). It is possible to distinguish several discernible phenotypes of bone marrow perivascular MSC by specific cell surface markers, underscoring distinct functional subpopulations. For instance, the presence or absence of Nestin (NES), neural/glial antigen (NG)2, leptin receptor (LepR), CD271, and CD146 discriminates perisinusoidal, periarteriolar, and endosteal MSC within bone marrow, all with different interactive properties with hematopoeitic stem cells at different stages (i.e., active vs. quiescent) and differentiation potentials (Sacchetti et al. 2007; Tormin et al. 2011; Mendelson and Frenette 2014). In addition to supporting hematopoiesis, their perivascular phenotype equips MSC with parallel physiological in vivo functions such as a "therapeutic" function during tissue injury, orchestrating immunomodulatory and trophic responses via secretion of paracrine signaling factors (Caplan and Dennis 2006; Singer and Caplan 2011; Caplan and Correa 2011; Bernardo and Fibbe 2013). The latter has been proposed as a common feature of MSC throughout the body, thus complementing their originally defined progenitor role for mesenchymal tissues, especially skeletal tissues. Consequently, the progenitor and "therapeutic"

capabilities of MSC have been heavily explored, giving rise to in vitro and in vivo protocols to treat skeletal conditions, based on concepts of tissue engineering and cell therapy.

7.2 Isolation and Characterization

Adult MSCs have an excellent capacity to repair tissues, they are potentially accessible in high quantities with low donor site morbidity, and due to their easy manipulation and good differentiation capacity, they are of high interest for cell-based and tissue engineering applications. The tissue sources for MSC involve primarily the bone marrow and the adipose tissue; however, phenotypically similar cells have also been isolated from intra- and periarticular tissues such as the superficial layer of the cartilage, synovium, periosteum, and infrapatellar fat pad, as well as from fetal (e.g., placenta, umbilical cord, and amniotic fluid) and other adult tissues (Williams et al. 2010; Koh and Choi 2012; Candela et al. 2014). MSCs are commonly isolated by plastic adherence or, when possible, by using specific cell surface markers. In vitro characterization of MSC was first proposed by Pittenger et al., including tri-lineage differentiation capacity (adipogenic, osteogenic, and chondrogenic) and expression of specific surface markers such as CD105, CD106, CD90, and CD73 (Pittenger et al. 1999). In parallel, together with Johnstone et al. and Muraglia et al., they also highlighted for the first time the heterogeneous nature of these cells demonstrated by the different multilineage differentiation capacity of MSC clones (Johnstone et al. 1998; Pittenger et al. 1999), which in turn can be influenced by adding exogenous growth factors to the culture medium (Muraglia et al. 2000). More recently, the International Society for Cellular Therapy (ISCT) added to the definition of MSCs as cells negative for the hematopoietic markers CD11b or CD14, CD19 or CD79a, CD34, and CD45 and for human leukocyte antigen (HLA)-DR (Dominici et al. 2006). More recently, CD146 was proposed as a potent marker for the selection of MSC with enhanced differentiation and colony-forming capacity (Sacchetti et al. 2007; Kaltz et al. 2010). Similarly, starting from 2002, CD271 has acquired a great interest as a tool for the isolation of MSC subpopulations with high chondrogenic potential (Quirici et al. 2002; Hermida-Gómez et al. 2011; Ghazanfari et al. 2016).

7.3 Expansion

MSC can be expanded in vitro; however, their proliferative capacity is limited and it parallels the reduction in their differentiation capacity (Banfi et al. 2000; Bonab et al. 2006). Therefore, looking for culture medium modifications to ameliorate the expansion capacity of MSC has been (and still is) a great challenge in the field. Taking inspiration from limb development, FGF2 was the first medium additive successfully used to enhance both MSC expansion and differentiation capacity (Martin et al. 1997), and it is still the stimulus most used for expansion of MSC in

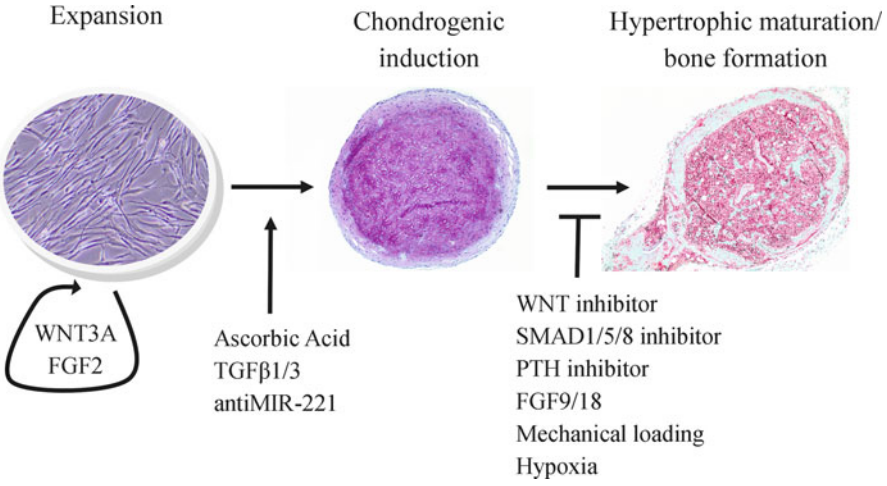


Fig. 8 MSC chondrogenic differentiation program. Following expansion and early chondrogenic differentiation, MSCs have the tendency to undergo terminal hypertrophic maturation in vitro and in vivo, reminiscent of endochondral ossification. In this schematic overview, we report some of the most relevant interventions that regulate the chondrogenic differentiation of these cells at various stages. Left panel, MSC during expansion (brightfield); middle panel, chondrogenic pellet (aggregate) culture (thionin staining); chondrogenic pellets after 12 weeks of in vivo implantation (hematoxylin/eosin staining)

vitro (Tsutsumi et al. 2001; Solchaga et al. 2010; Narcisi et al. 2015). Nevertheless, the mechanism of action of FGF2 is still poorly understood (Ito et al. 2007; Handorf and Li 2011), and it has been demonstrated that its in vitro effect does not necessarily predict its in vivo activity (Sacchetti et al. 2007). More recent work has shown that the combination of FGF2 with WNT3A further improves long-term expansion and chondrogenic capacity both in vitro and in vivo (Narcisi et al. 2015) (Fig. 8). Interestingly, the administration of WNT protein alone can either stimulate by the canonical Wnt pathway or inhibit by the noncanonical pathway the proliferation of MSC, with remarkable different effects on differentiation capacity (de Boer et al. 2004; Baksh et al. 2007). Another factor extensively studied is TGF-β, also with reported conflicting results. Sawada et al., for example, demonstrated that TGF-β signaling can promote cellular senescence of MSC by inducing their cell cycle arrest via activation of p16, p21, and p53 proteins (Sawada et al. 2006), while other authors reported that TGF-β stimulates proliferation of MSC (Jian et al. 2006). In agreement with the last work, a serum-free medium mainly composed of TGF-β, FGF2, and PDGF was proposed as an effective expansion medium for MSC (Chase et al. 2010). The potential of PDGF in stimulating proliferation, while maintaining the differentiation potential of the cells, is known for MSC derived from different tissues (Fierro et al. 2007; Mizuno et al. 2015). PDGF is also one of the main components of platelets and plasma derivatives, products that in the last years are acquiring growing interest in the scientific community for their proliferation inductive properties and for sustaining the differentiation capacity of MSC (Doucet et al. 2005; Fekete et al.

2014; Muraglia et al. 2015). In order to provide new systems to optimize harvesting and expansion of MSC, and to enhance their applicability into the clinic, new approaches have been developed recently for the selection and the expansion of MSC in a "3D" environment under direct perfusion (Papadimitropoulos et al. 2014), in scaffolds designed with the 3D printers (Ferlin et al. 2016), or by combining biodegradable nanofibers and platelet-rich plasma (Diaz-Gomez et al. 2014). These new approaches combined with an increasing knowledge in the cell biology of MSC are positively contributing to the transition of MSC to the clinic. However, the donor-to-donor variability is still an unresolved problem, and the scientific community should strive to find new solutions to obtain more homogeneous populations of MSC.

7.4 Chondrogenic Differentiation

Chondrogenic differentiation of MSC is classically performed in aggregate (pellet) culture, and, mimicking the cartilage development, it proceeds via three main stages: condensation, differentiation, and hypertrophy/terminal differentiation. Each of the three stages is characterized by the expression of specific markers, such as N-cadherin for the condensation, collagen type II and aggrecan for the differentiation, and collagen type X and alkaline phosphatase (ALP) for the hypertrophy/terminal differentiation stage (Barry et al. 2001; Hellingman et al. 2011b). In 1998, Johnstone et al. proposed for the first time the use of TGF-β as the essential pro-chondrogenic factor for the differentiation of MSC. For stable cartilage formation, adaptations to this protocol were proposed in order to limit the tendency of MSC to undergo terminal differentiation. This has been done mainly by interfering with signaling by parathyroid hormone (PTH) (Weiss et al. 2010), WNT (Narcisi et al. 2015), TGF-β (Hellingman et al. 2011b), or different FGF ligand proteins during the different chondrogenic differentiation stages (Correa et al. 2015) (Fig. 8). The intracellular cross talk among WNT, TGF-β, and FGF signaling pathways is particularly complex and is still studied extensively for their importance during development (Cleary et al. 2015). Other alternative methods to preserve cartilage formation and limit hypertrophy of MSC have been proposed, including mechanical stimulation in several different 3D models (Panadero et al. 2016; Steward et al. 2016; Gardner et al. 2016) and the differentiation of MSCs under hypoxic conditions (Shang et al. 2014; Leijten et al. 2014), in the attempt to reproduce the natural cartilage environment (Fig. 8).

As an alternative to the aggregate cultures, several other systems are available to test the chondrogenic capacity of MSC in vitro. Of note is the development of osteochondral models that attempt to better mimic the in vivo environment, both physiologically and pathologically (de Vries-van Melle et al. 2014; Barron et al. 2015). Similarly, various groups are developing systems that can induce MSC differentiation toward the chondrogenic lineage in the absence of exogenously administered growth factors. Particularly fascinating is how simple modifications in surface topography (Kilian et al. 2010), cell geometry (Lee et al. 2013), silencing single anti-chondrogenic factors (Lolli et al. 2016), and blocking the presence of a

pro-angiogenic molecule (Nagai et al. 2010; Marsano et al. 2016) can "spontaneously" direct the chondrogenic differentiation of MSC. What is still missing is a detailed stage-specific study that could allow a better specification and characterization of the different chondrogenic phases (condensation, differentiation, and hypertrophy/terminal differentiation), possibly providing the development of stage-based strategies and therefore a more tailored culture regimes in which cells are exposed transiently at different stages to the optimal signals or even "primed" in a way to allow them to acquire naturally the correct phenotype when transferred in vivo.

Although most studies focus on the generation of articular cartilage, MSC could be a source for nasal and auricular cartilage generation. Zhao et al. have recently reported on chondrodifferentiation of sheep bone marrow MSCs into an auricular chondrocyte lineage using medium conditioned by auricular chondrocytes, with and without TGF-β3 (Zhao et al. 2016). The results from that study showed that the auricular chondrocyte-conditioned medium alone provides a significant stimulus to chondrodifferentiate the cells to an auricular chondrocyte phenotype complete with the expression of elastin in the ECM. MSCs have also achieved promising results in meniscus tissue engineering (reviewed by Niu et al. 2016), alone or after co-culture with MCs in ratios ranging from 1:19 to 3:1 (Cui et al. 2012; Matthies et al. 2012; Kremer et al. 2017); however research is still ongoing to determine the ideal cell types and cell rations and to optimize the clinical application. Taken together, these studies suggest that soluble factors from chondrocytes can be employed to differentiate MSC down a specified chondrogenic pathway.

7.5 Preclinical Studies

Two main therapeutic approaches have been developed for the use of MSC to treat cartilage defects, based on distinct ultimate purposes. While tissue engineering protocols exploit the differentiation potential of MSC, aiming at restoring the architecture and function of the tissue either in vivo or ex vivo (Caplan 2007), cell-based therapy takes advantage of the immunomodulatory and trophic activities of MSC to establish a local microenvironment conducive to in situ tissue regeneration (Caplan and Correa 2011). As a consequence, several animal models have been developed to establish proof-of-concept regarding the feasibility of using MSC from various sources (e.g., bone marrow, adipose tissue, synovium, muscle) in the context of both cartilage tissue engineering and cell-based therapy. These include small animals (e.g., rats, rabbits) (Koga et al. 2008; Dashtdar et al. 2011), as well as large animal models (e.g., sheep, goats, horses, and pigs) (McIlwraith et al. 2011; Bekkers et al. 2013), in which the intervention to inflict the tissue damage is essentially different.

Cartilage tissue engineering techniques are designed primarily for the repair of chondral (partial and full thickness) and osteochondral defects, generated mainly at the femoral condyles and the femoral trochlea. The cells can be injected directly into

the joint or as part of a tissue-engineered construct (TEC) implanted into the defect (Kon et al. 2015; Huang et al. 2016). These TEC are typically made by a tridimensional scaffold (synthetic or natural), in which the MSCs (primarily from bone marrow) are pre-seeded and guided for differentiation by specific "cocktails" of signaling factors (Klein et al. 2009; Kock et al. 2012). Combinations of these three components have been extensively used, incorporating additional steps such as in vivo marrow stimulation, addition of biological sealants or viscosupplementation, and in vitro culture and preconditioning for 2–4 weeks (e.g., bioreactor-based mechanical stimulation) prior to implantation (Pei et al. 2002; Grayson et al. 2010; Gadjanski et al. 2012). In general, single intra-articular injections of MSC alone, independent from their source, fall short of rebuilding hyaline cartilage, as suboptimal fibrocartilage tissue forms to resurface the defects (Koga et al. 2008). In contrast, serial injections and direct implantation of bone marrow-derived MSC into the defect, in combination with hyaluronic acid (HA) or microfracture, have shown better results in some reports, based on tissue integration and proteoglycan and type II collagen contents (Lee et al. 2007; Saw et al. 2009; Nam et al. 2013). On the other hand, various TEC variations have been investigated in preclinical settings. Both scaffold-free and MSC-seeded biodegradable matrices have been tested for chondral and osteochondral defects with variable success rates. The results show a tendency to resurface the defects with a hyaline-like cartilage, which integrates better with native surrounding tissue, and underlying bone regeneration in osteochondral defects (reviewed in (Bornes et al. 2014)). A strategy that seems to improve the quality of the repair tissue, evident by macroscopic and histological scores, is the exposure of the developing TEC to a culture period (2 to 3 weeks) (Zscharnack et al. 2010; Marquass et al. 2011). During this pre-implantation period, MSCs are exposed to pro-differentiating conditions inside the scaffold and subjected to preconditioning in bioreactors that mimic the mechanical environment the construct experiences later in vivo (Khozoee et al. 2016) (examples were discussed above in Sects. 7.3 and 7.4). Lastly, efforts have been focused on determining the benefits of mixing freshly isolated chondrons (articular cartilage chondrocytes in their own ECM) with expanded MSC to treat focal defects. The rationale for this approach is twofold: first, it solves the low cell counts typically obtained with chondrocytes alone (without expansion), thus permitting a single-stage procedure instead of a two-step approach (Bekkers et al. 2013); and second, a bidirectional cross talk between the two cell types has been demonstrated, which brings synergistic benefit to both cell types including proliferation and stabilization of the phenotype (Acharya et al. 2012).

A cell-based therapy approach, on the contrary, is designed mostly for diffuse, non-focal erosion of the tissue, typically due to lifelong degenerative changes (e.g., osteoarthritis). The overall consensus in all these studies is the lack of solid evidence of directed chondrogenic differentiation of injected MSC (all sources) that restores the tissue volume. Rather, they have established the notion of a paracrine activity of MSC that influences the local environment, including responses from local progenitors (e.g., from synovium), slowing down the degenerative process, partially restoring the tissue architecture, reducing subchondral sclerosis, and remodeling

associated osteophytes (Frisbie et al. 2009; Toghraie et al. 2011, 2012; Sato et al. 2012; Faqeh et al. 2012; Horie et al. 2012; Huurne et al. 2012; Diekman et al. 2013). Interestingly, when meniscectomy is performed, a meniscal regenerative response has been documented. Again, the resulting regenerated tissue consists mostly of host cells with only few transplanted cells (Murphy et al. 2003).

7.6 Clinical Studies

A therapeutic logic similar to the one applied for preclinical studies, incorporating tissue engineering and cell-based therapy approaches, is exercised for human clinical trials. In that sense, focal defects (chondral and osteochondral) are primarily managed with engineered constructs (i.e., TEC), while more widespread damage (e.g., OA) is addressed by cell-based therapy. So far, the use of MSC as part of a TEC to treat focal defects has been reduced exclusively to bone marrow-derived cells (Bornes et al. 2014; Kon et al. 2015). Their superior chondrogenic differentiation potential compared with other sources (e.g., adipose tissue) and long-term safety has supported their clinical investigative use in chondral defects in the femoral condyles and trochlea, tibial plateau, patella, and talus, as well as in osteochondral defects generated, for instance, by osteochondritis dissecans (Teo et al. 2013). MSCs combined with chondrocytes or chondrons have been used clinically, as well (de Windt et al. 2016). It is proposed that the positive effects seen in this procedure are due to the paracrine effects of MSC rather than their differentiation capacity.

As with preclinical studies, MSC can be implanted alone or seeded in biological (e.g., collagen gels or membranes, HA, fibrin-rich gels or glues) or combinations of biological and synthetic (e.g., PGA-HA) matrices. The TEC can be implanted right after cell seeding or after a period of in vitro culture and preconditioning to promote adherence of the cells and their chondrogenic induction (Wakitani et al. 2007; Kuroda et al. 2007) and covered by periosteal or synovial flaps to stabilize the construct within the implanted site. The overall clinical results are positive when evaluated up to 24 months, in terms of functional scales, imaging (MRI), and direct observation through arthroscopy evaluating parameters such as integration, surface, and filling (summarized in Bornes et al. (2014)). Unfortunately, the literature is limited regarding longer evaluation periods, especially showing efficacy beyond safety. When biopsies are performed, a mixture of hyaline-like and fibrocartilage is found with signs of circumscribed hypertrophy, in some cases covering healthy tissue (Giannini et al. 2009).

More generalized cartilage damage, like that generated by OA, requires a different therapeutic approach. In such approach, it is paramount to modulate the pathological environment driving the degenerative changes rather than just locally replacing chondrocytes and ECM. Encouraged by the recent findings related to the anti-inflammatory, trophic, and immunomodulatory effects of MSC (Caplan and Correa 2011), stem cell-based therapies to manage OA have gained enthusiasm and attention. Various cell sources (e.g., adipose tissue, bone marrow, infrapatellar fat pad, synovium, umbilical cord, etc.) have been used clinically, by intra-articular

injection after expansion, alone or in combination with collagen gels or platelet-rich plasma (PRP), and at highly variable cell concentrations (Gupta et al. 2012; Burke et al. 2016). Even though most reported studies are open-label, single-arm trials (e.g., Orozco et al. (2013); Soler et al. (2016)), initial evidence has pointed to superior clinical effects of MSC at 12 months or more compared with standard treatments (Koh and Choi 2012) or injected HA (Lamo-Espinosa et al. 2016), based on both clinical scores (e.g., WOMAC, KOOS, VAS, etc.) and imaging analysis (e.g., X-rays, MRI, and T2 mapping). Recently, autologous adipose-derived stromal vascular fraction (SVF) has been introduced as an option, with or without PRP mixed during the injection (www.clinicaltrials.gov, NCT02726945, NCT02846675, NCT01947348). In parallel, there are an increasing number of randomized, double-blinded, placebo-controlled phase II clinical trials studying a longer effect of culture-expanded bone marrow or adipose-derived MSC on OA lesions, testing various cell doses. These clinical data will be crucial to unravel the ultimate clinical benefit of a cell-based therapy for OA, bearing in mind the necessity to control for the standard, currently used therapies.

8 Pluripotent Stem Cell-Derived Chondrocytes

As a potential noninvasive cell source for a large amount of chondrocytes, pluripotent stem cells (PSCs) have become highly attractive for cartilage regeneration. This field currently lacks an optimal cell source, because the only applicable cells – human articular chondrocytes – are extremely limited in supply. Although mesenchymal stromal cells from bone marrow (BMSC) or adipose tissue have the possibility of overcoming cell shortage, they are somewhat committed to the endochondral pathway and often inadvertently form bone in vivo. PSCs promise to overcome both limitations, since they possess a virtually unlimited expansion capacity and are intrinsically capable of forming any adult tissue including stable hyaline cartilage.

8.1 Isolation and Characterization

Physiologically, PSCs form the inner mass of the blastocyst and are, thus, the origin of all embryonic tissues. By sacrificing the fertilized embryo, the inner cell mass can be isolated immunosurgically and cultured as a stable cell line termed embryonic stem cells (ES cells) (Thomson et al.1998). Of note, the ethical controversy of this step has led to legal constrictions for ES cell research. Alternatively, PSCs can be directly generated from any nucleated somatic cell source by forced expression of a small set of transcription factors that induce reprogramming of the cellular differentiation status back to pluripotency. The originally used reprogramming factors for generating such induced pluripotent stem cells (iPS cells) comprised octamer-binding transcription factor (OCT)3/4, SOX2, Kruppel-like factor (KLF)4, and myelocytomatosis oncogene (cMYC) (Takahasi et al. 2006), and various additions

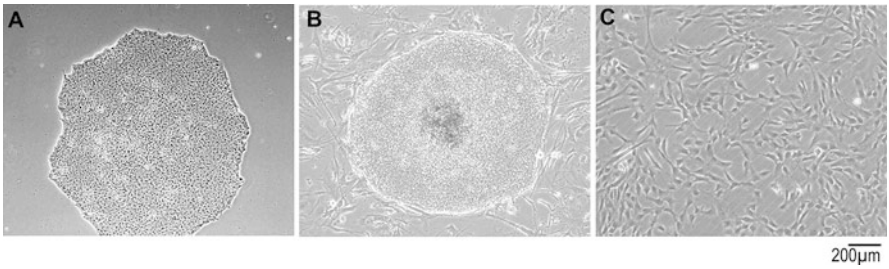


Fig. 9 Cell morphology of PSCs and MPCs. (**a**) Typical iPS cell colony (line iPS-D1 (Larribere et al. 2015)) cultured feeder-free on Matrigel®. (**b**) iPS cell colony (line 48 M–iPS-1) on mouse embryonic fibroblasts. (**c**) MPCs generated from 48 M–iPS-1 cells (Diederichs and Tuan 2014)

such as microRNA and small molecule-enhanced approaches have been reported since (Judson et al. 2009; Ma et al. 2013).

PSCs in culture form dense colonies of non-discernible small individual cells (Fig. 9). Essential characteristics of PSCs are prolonged undifferentiated proliferation in vitro (self-renewal) and stable differentiation capacity to form derivatives of all three embryonic germ layers (Thomson et al. 1998). This developmental potential can be tested in vitro by inducing PSCs to express germ layer-specific markers when differentiated into embryoid bodies – small cell aggregates in which tissues from all three germ layers are formed spontaneously, similar to the process in the embryo, but far less coordinated. Mature tissues, however, can currently be obtained only in teratomas formed by PSCs in vivo. Contribution to the germ line in chimeras and generation of all PSC offspring are certainly the most stringent functional tests for PSCs, but these cannot be performed with human cells for ethical and practical reasons (Cahan and Daley 2013). Although there is no appropriate replacement for functional testing, the detection of pluripotency-associated markers and the epigenetic and gene expression profiling are frequent quality assessments.

8.2 Expansion

PSCs were originally cultured on feeder layers of mitotically inactivated mouse embryonic fibroblasts (Fig. 9b), which provide essential growth factors and a substrate (Thomson et al. 1998). To avoid contamination with mouse fibroblasts, PSC lines can often be adapted to feeder-free culture on ECM proteins (e.g., Matrigel®, Geltrex®, laminin, vitronectin, fibronectin) when treated with conditioned medium or with defined medium containing a high FGF2 concentration (100 ng/mL) in combination with TGF-β or the TGF-β superfamily member Nodal (Ludwig et al. 2006; Chen et al. 2011). High nutrient consumption requires daily medium exchanges, and the tendency to spontaneously differentiate under slightly suboptimal conditions often requires manual removal of such colonies in order to maintain the overall undifferentiated culture. Moreover, PSCs are passaged as aggregates (Thomson et al. 1998) and survive poorly when dissociated into single

cells. Inhibition of Rho-associated protein kinase is frequently used to increase PSC survival after passaging and to allow clonal expansion (Watanabe et al. 2007). In consequence, while inherently capable of yielding infinite cell numbers, obtaining large amounts of PSCs is highly elaborate and costly. To overcome this issue, novel biotechnological techniques including automated large-scale culture and suspension culture in bioreactors are currently being adapted for scaling up PSC culture (Olmer et al. 2012; Haraguchi et al. 2015; McLaren et al. 2013).

8.3 Chondrogenic Differentiation

Recapitulating embryonic cartilage development in vitro is currently considered the most promising strategy in order to differentiate PSCs into mature cartilage-forming chondrocytes (Oldershaw et al. 2010). Thus, cells are sequentially passed through consecutive developmental stages including intermediate meta-stable mesenchymal progenitors, which are subsequently induced into the chondrogenic lineage. To generate such mesenchymal progenitor cells (MPCs), a frequently applied method is to withdraw the pluripotency-supporting culture conditions and to culture the adherent cells in serum-containing medium until they acquire the mesenchymal phenotype (Diederichs and Tuan 2014; Frobel et al. 2014). Alternatively, MPCs can be established from the adherent cells growing out of embryoid bodies (Diederichs and Tuan 2014).

Subsequently, cartilage-forming chondrocytes can be generated by treatment of MPC micromass cultures with TGF-β in chondrogenic medium (Fig. 10) (Diederichs and Tuan 2014; Frobel et al. 2014). However, cartilage formation by MPCs is less efficient than with chondrocytes or BMSC, probably at least partly caused by the insufficient induction of the master chondrogenic transcription factor SOX9 (Diederichs et al. 2016). The addition of FGF2, PDGF, GDF5, or BMPs (e.g.,

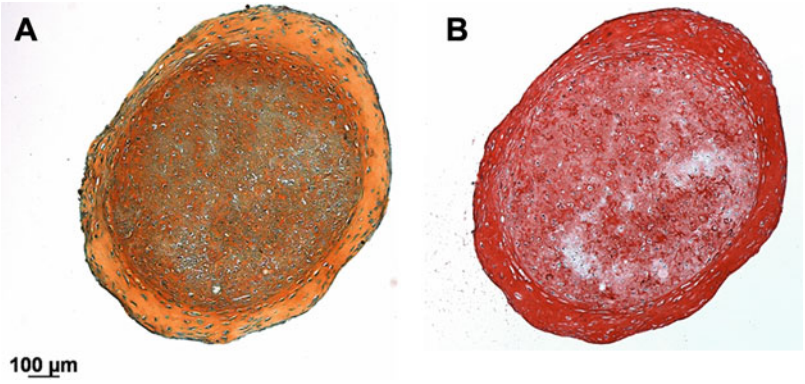


Fig. 10 In vitro cartilage formation of PSCs. Safranin O staining of proteoglycans (**a**) and immunohistological staining of collagen type II (**b**) indicate formation of cartilage from MPCs after 6 weeks of chondrogenic pellet culture with TGF-β

BMP2, BMP4, BMP6) has been suggested, but a consensus protocol has not yet been found. BMPs, however, should be used with care, since they can co-induce undesired endochondral ossification (Diederichs et al. 2016).

Moreover, sophisticated MPC generation strategies using more stringent mesodermal stimulation have been suggested with the aim of increasing MPC homogeneity and cartilage formation. Growth factor treatment (canonical Wnt agonists, Activin A, etc.) complemented by cell enrichment (via surface markers or manual isolation of cell condensates) and subsequent chondrogenic micromass culture may allow chondrogenesis (Oldershaw et al. 2010; Umeda et al. 2012; Yamashita et al. 2015). Using such sophisticated strategies, PSC-derived cartilage has been reported to be histologically positive for proteoglycans and collagen II similarly as MSC-derived cartilage but remained negative for the fibrous collagen I (Umeda et al. 2012; Yamashita et al. 2015). The next step after these first highly promising results will be to simplify the current differentiation strategies and to improve their efficiency in order to yield high numbers of active chondrocytes with feasible efforts and costs. The key will be to either find factors stringent enough to force the complete PSC population into homogeneous differentiation or to develop culture conditions that automatically enrich for specific subpopulations – for example, by size exclusion of cell condensates – without manual intervention or sorting.

8.4 Applications

Ambitions to make PSCs clinically applicable are spurred by such fields where PSCs are the only available cell source (e.g., neurobiology, cardiology, etc.), and first clinical studies have been initiated for the treatment of macular degeneration, spinal cord injury, and type I diabetes [recently reviewed by Takahashi and Yamanaka (2016)]. Cartilage regeneration can greatly benefit from these fields rendering possible the future application of PSC-derived chondrocytes and cartilage tissue in cell-based cartilage therapy. Indeed, significant progress has already been achieved to make PSCs clinically safe. Stringent purification strategies and suicide genes have been proposed to eliminate remnant PSCs that could potentially form tumors upon implantation when they proliferate in an uncontrolled manner. A remaining challenge is a considerable PSC variability. This can arise from a number of causes including variable presence of pre-existing mutations in the originating cells, newly arising mutations and changes of gene copy number during reprogramming or prolonged culture, incomplete reprogramming and retained epigenetic memory, as well as aberrant reprogramming of DNA methylation and imprinting (Gore et al. 2011; Lister et al. 2011; Laurent et al. 2011; Nazor et al. 2012). Continuously advancing techniques for the assessment of the global genetic, epigenetic, and metabolic cell profile will enable establishing sophisticated monitoring strategies to ensure reproducible PSC quality.

Of note, the current perception in the field tends toward allogeneic strategies, because deriving autologous PSCs either by reprogramming into iPS cells or by isolating ES cells from an embryo generated by somatic cell nuclear transfer is a

highly time-consuming process. Although in principle any nucleated cell is reprogrammable, young cells seem preferable over adult cells as origin. During their short life time, fetal cells have accrued less DNA damages and somatic mutations that would persist upon reprogramming (Gore et al. 2011). Cord blood and other perinatal cells are thus highly attractive for reprogramming, and iPS cell banks are currently being set up in several countries (Turner et al. 2013). As a consequence, for PSC-derived cartilage to enter clinical application, many years of preclinical and clinical studies are still required.

The present value of PSCs therefore lies in the development of genetic disease models to illuminate unknown pathomechanisms and for drug screening. PSCs can recapitulate human diseases much better than immortalized human cell lines and also allow investigation of tissue formation in vivo upon implantation into experimental animals. Thus, PSCs already are a valuable complementary strategy for animal models, e.g., for the innumerous osteochondral dysplasias. While the disease-causing mutations for many of these conditions are known, it remains unclear how, for example, defective collagen or constitutively active FGF receptors cause the highly diverse symptoms and how these can be treated effectively. Using iPS cell models, statins have been suggested as a novel treatment option for chondrodysplasias (Yamashita et al. 2014), a reason for the failure of anti-IL-1beta therapy in neonatal-onset multisystem inflammatory disease (NOMID) was discovered (Yokoyama et al. 2015), and cell stress was identified as central issue in type II collagenopathy (Okada et al. 2015).

In summary, the first promising results have already been achieved with regard to in vitro generation of hyaline cartilage from human PSCs, but robust, reproducible, and simple methods need to be developed. PSC-derived chondrocytes and cartilage promise to overcome the limited supply of primary chondrocytes and the apparent inability of BMSC to generate stable cartilage. While clinical application still requires years of development, novel in vitro models of genetic diseases have already yielded new insights into pathomechanisms and enable high-throughput drug screening in human cells and even drug testing in human tissues in vivo.

9 Summary and Conclusion

Cartilage at all sites of our body has limited intrinsic capacity to repair. There are numerous methods to use when repairing cartilage, and the preferred method depends on the location of the cartilage defect, whether it is the nose, the ear, the airway, or a certain site in the joint. The application of techniques using different chondrogenic cells to fill the defect areas to start a process of chondrogenesis and subsequent cartilage repair is becoming more common, in particular for joint cartilage defect repair. Different cell types with chondrogenic capacities are being used, from MSC to committed chondrocytes. Today, there is no proof that one cartilage repair method is clearly better than the others.

Obtaining cartilage of good quality using cell-based repair approaches is challenging. Next to the tissue being too fibrous, it can become hypertrophic and form

bone. Much knowledge can be obtained from embryology. The separate embryonic origin of articular chondrocytes and transient chondrocytes, which prefigure the bony skeleton, might be associated with more profound differences in chondrogenesis than previously assumed and explain why redifferentiation of in vitro expanded, dedifferentiated articular chondrocytes can yield hyaline cartilage, while chondrogenically differentiated MSCs undergo premature hypertrophy and mineralize. Similarly, chondrocytes from different cell sources, even from the same cartilage type, vary in cell behavior, growth, ECM production, and mechanical properties, tending to maintain their particular developmental characteristics and exhibit differences in gene expression despite culture expansion (Chung et al. 2008; Hellingman et al. 2011a; Ishizeki et al. 2003; Isogai et al. 2006; Kusuhara et al. 2009; Leijten et al. 2012; ElSayed et al. 2010).

Much knowledge on cell behavior can be obtained using chondrogenic cell lines. Although they are not suitable for use for clinical applications, they are useful as model systems to study basic mechanisms of cartilage repair and disease. The more recently developed PSC-based technology may allow generation of in vitro models that recapitulate genetic human diseases more realistically than immortalized cell lines. Thus, they promise to become an invaluable complementation to animal models allowing high-throughput drug screening as well as elucidation of currently unknown pathomechanisms. Whether PSCs will be able to live up to the expectations for application in cell therapy will depend on our ability to generate large amounts of clinically safe PSCs and to differentiate them into regeneratively active cells like chondrocytes with acceptable efforts.

Acknowledgments Andrea Barbero is supported by the Swiss National Science Foundation (SNSF Project No. 310030_149614) and the Deutsche Arthrose-Hilfe (P245-A546-Martin-EP1-mumm1-knie-op-III-2011-15).

Diego Correa is in gratitude with the Case Center for Multimodal Evaluation of Engineered Cartilage (CCMEEC) through the NIH grant P41-EB021911, the Soffer Family Foundation, and the Diabetes Research Institute Foundation (DRIF).

Tim Hardingham in the Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, acknowledges support from the Wellcome Trust (grant number 088785/Z/09/Z).

Roberto Narcisi is supported by a VENI grant by STW (nr. 13659).

Mark Randolph would like to acknowledge Irina Pomerantseva, M.D., Ph.D., and Cathryn A. Sundback, Sc.D., from the Laboratory for Tissue Engineering and Organ Fabrication, Massachusetts General Hospital, for their contributions on the biology of auricular chondrocytes for generating ear cartilage.

Sandra van den Bosch is acknowledged for help with editing of the references.

References

Acharya C, Adesida A, Zajac P, Mumme M, Riesle J, Martin I, Barbero A (2012) Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in coculture pellets mediate improved cartilage formation. J Cell Physiol 227:88–97

Akgun I, Unlu MC, Erdal OA, Ogut T, Erturk M, Ovali E, Kantarci F, Caliskan G, Akgun Y (2015) Matrix-induced autologous mesenchymal stem cell implantation versus matrix-induced

autologous chondrocyte implantation in the treatment of chondral defects of the knee: a 2-year randomized study. Arch Orthop Trauma Surg 135:251–263

Akihiro Yamashita, Miho Morioka, Yasuhito Yahara, Minoru Okada, Tomohito Kobayashi, Shinichi Kuriyama, Shuichi Matsuda, Noriyuki Tsumaki, (2015) Generation of Scaffoldless Hyaline Cartilaginous Tissue from Human iPSCs. Stem Cell Reports 4 (3):404–418

Akihiro Yamashita, Miho Morioka, Hiromi Kishi, Takeshi Kimura, Yasuhito Yahara, Minoru Okada, Kaori Fujita, Hideaki Sawai, Shiro Ikegawa, Noriyuki Tsumaki, (2014) Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature 513 (7519):507–511

Aksoy F, Yildirim YS, Demirhan H, Ozturan O, Solakoglu S (2012) Structural characteristics of septal cartilage and mucoperichondrium. J Laryngol Otol 126:38–42

Allen Kuan-Liang Chen, Xiaoli Chen, Andre Boon Hwa Choo, Shaul Reuveny, Steve Kah Weng Oh, (2011) Critical microcarrier properties affecting the expansion of undifferentiated human embryonic stem cells. Stem Cell Research 7 (2):97–111

Alexander TH, Sage AB, Chen AC, Schumacher BL, Shelton E, Masuda K, Sah RL, Watson D (2010) Insulin-like growth factor-I and growth differentiation factor-5 promote the formation of tissue-engineered human nasal septal cartilage. Tissue Eng Part C Methods 16:1213–1221

Alexander TH, Sage AB, Schumacher BL, Sah RL, Watson D (2006) Human serum for tissue engineering of human nasal septal cartilage. Otolaryngol Head Neck Surg 135:397–403

Attur MG, Dave MN, Clancy RM, Patel IR, Abramson SB, Amin AR (2000) Functional genomic analysis in arthritis-affected cartilage: yin-yang regulation of inflammatory mediators by alpha 5 beta 1 and alpha V beta 3 integrins. J Immunol 164:2684–2691

Aulthouse AL, Beck M, Griffey E, Sanford J, Arden K, Machado MA, Horton WA (1989) Expression of the human chondrocyte phenotype in vitro. In Vitro Cell Dev Biol 25(7):659–668

Baksh D, Boland GM, Tuan RS (2007) Cross-talk between Wnt signaling pathways in human mesenchymal stem cells leads to functional antagonism during osteogenic differentiation. J Cell Biochem 101:1109–1124

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R (2000) Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. Exp Hematol 28:707–715

Barandun M, Iselin LD, Santini F, Pansini M, Scotti C, Baumhoer D, Bieri O, Studler U, Wirz D, Haug M, Jakob M, Schaefer DJ, Martin I, Barbero A (2015) Generation and characterization of osteochondral grafts with human nasal chondrocytes. J Orthop Res 33:1111–1119

Barbero A, Ploegert S, Heberer M, Martin I (2003) Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 48:1315–1325

Barron V, Merghani K, Shaw G, Coleman CM, Hayes JS, Ansboro S, Manian A, O'Malley G, Connolly E, Nandakumar A, van Blitterswijk CA, Habibovic P, Moroni L, Shannon F, Murphy JM, Barry F (2015) Evaluation of cartilage repair by mesenchymal stem cells seeded on a PEOT/PBT scaffold in an osteochondral defect. Ann Biomed Eng 43:2069–2082

Barry F, Boynton RE, Liu B, Murphy JM (2001) Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 268:189–200

Bartlett W, Skinner JA, Gooding CR, Carrington RW, Flanagan AM, Briggs TW, Bentley G (2005) Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. J Bone Joint Surg Br 87:640–645

Basad E, Ishaque B, Bachmann G, Stürz H, Steinmeyer J (2010) Matrix-induced autologous chondrocyte implantation versus microfracture in the treatment of cartilage defects of the knee: a 2-year randomised study. Knee Surg Sports Traumatol Arthrosc 18:519–527

Bauer BS (2009) Reconstruction of microtia. Plast Reconstr Surg 124:14e–26e

Bekkers JE, Tsuchida AI, van Rijen MH, Vonk LA, Dhert WJ, Creemers LB, Saris DB (2013) Single-stage cell-based cartilage regeneration using a combination of chondrons and mesenchymal stromal cells: comparison with microfracture. Am J Sports Med 41:2158–2166

Benoit B, Thenet-Gauci S, Hoffschir F, Penformis P, Demignot S, Adolphe M (1995) SV40 large T antigen immortalization of human articular chondrocytes. In: In Vitro Cell Dev Biol, vol 31, pp 174–177

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, Shaffer JD (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224

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

Bermueller C, Schwarz S, Elsaesser AF, Sewing J, Baur N, BA v, Scheithauer M, Notbohm H, Rotter N (2013) Marine collagen scaffolds for nasal cartilage repair: prevention of nasal septal perforations in a new orthotopic rat model using tissue engineering techniques. Tissue Eng Part A 19:2201–2214

Bernardo ME, Fibbe WE (2013) Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell 13:392–402

Bernhard JC, Vunjak-Novakovic G (2016) Should we use cells, biomaterials, or tissue engineering for cartilage regeneration? Stem Cell Res Ther 7:9

Bernier SM, Goltzman D (1993) Regulation of expression of the chondrogenic phenotype in a skeletal cell line (CFK2) in vitro. J Bone Miner Res 8:475–484

Bichara DA, Zhao X, Hwang NS, Bodugoz-Senturk H, Yaremchuk MJ, Randolph MA, Muratoglu OK (2010) Porous poly(vinyl alcohol)-alginate gel hybrid construct for neocartilage formation using human nasoseptal cells. J Surg Res 163:331–336

Bichara DA, Pomerantseva I, Zhao X, Zhou L, Kulig KM, Tseng A, Kimura AM, Johnson MA, Vacanti JP, Randolph MA, Sundback CA (2014) Successful creation of tissue-engineered autologous auricular cartilage in an immunocompetent large animal model. Tissue Eng Part A 20:303–312

Bleuel J, Zaucke F, Brüggemann GP, Niehoff A (2015) Effects of cyclic tensile strain on chondrocyte metabolism: a systematic review. PLoS One 10(3):25

Block JA, Inerot SE, Gitelis S, Kimura JH (1991) Synthesis of chondrocytic keratan sulphate-containing proteoglycans by human chondrosarcoma cells in long-term cell culture. J Bone Joint Surg Am 73(5):647–658

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

Bornes TD, Adesida AB, Jomha NM (2014) Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: a comprehensive review. Arthritis Res Ther 16:321–319

Brighton CT, Lorich DG, Kupcha R, Reilly TM, Jones AR, Woodbury RA 2nd (1992) The pericyte as a possible osteoblast progenitor cell. Clin Orthop Relat Res 287–299

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 31:889–895

Bujia J, Pitzke P, Kastenbauer E, Wilmes E, Hammer C (1996) Effect of growth factors on matrix synthesis by human nasal chondrocytes cultured in monolayer and in agar. Eur Arch Otorhinolaryngol 253:336–340

Bujia J, Sittinger M, Wilmes E, Hammer C (1994) Effect of growth factors on cell proliferation by human nasal septal chondrocytes cultured in monolayer. Acta Otolaryngol 114:539–543

Burke J, Hunter M, Kolhe R, Isales C, Hamrick M, Fulzele S (2016) Therapeutic potential of mesenchymal stem cell based therapy for osteoarthritis. Clin Transl Med 5:1–8

Candela ME, Yasuhara R, Iwamoto M, Enomoto-Iwamoto M (2014) Resident mesenchymal progenitors of articular cartilage. Matrix Biol 39:44–49

Candrian C, Vonwil D, Barbero A, Bonacina E, Miot S, Farhadi J, Wirz D, Dickinson S, Hollander A, Jakob M, Li Z, Alini M, Heberer M, Martin I (2008) Engineered cartilage generated by nasal chondrocytes is responsive to physical forces resembling joint loading. Arthritis Rheum 58:197–208

Cao Y, Rodriguez A, Vacanti M, Ibarra C, Arevalo C, Vacanti CA (1998) Comparative study of the use of poly(glycolic acid), calcium alginate and pluronics in the engineering of autologous porcine cartilage. J Biomater Sci Polym Ed 9:475–487

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650

Caplan AI (2008) All MSCs are pericytes? Cell Stem Cell 3:229–230

Caplan AI (2007) Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 213:341–347

Caplan AI, Correa D (2011) The MSC: an injury drugstore. Cell Stem Cell 9:11–15

Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076–1084

Chang AA, Reuther MS, Briggs KK, Schumacher BL, Williams GM, Corr M, Sah RL, Watson D (2012) In vivo implantation of tissue-engineered human nasal septal neocartilage constructs: a pilot study. Otolaryngol Head Neck Surg 146:46–52

Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC (2010) A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem Cell Res Ther 1:8

Cheng A, Hardingham TE, Kimber SJ (2014) Generating cartilage repair from pluripotent stem cells. Tissue Eng Part B Rev 20:257–266

Chua KH, Aminuddin BS, Fuzina NH, Ruszymah BH (2005) Insulin-transferrin-selenium prevent human chondrocyte dedifferentiation and promote the formation of high quality tissue engineered human hyaline cartilage. Eur Cell Mater 9:58–67; discussion 67:58–67

Chung C, Erickson IE, Mauck RL, Burdick JA (2008) Differential behavior of auricular and articular chondrocytes in hyaluronic acid hydrogels. Tissue Eng Part A 14:1121–1131

Cigan AD, Nims RJ, Vunjak-Novakovic G, Hung CT, Ateshian GA (2016) Optimizing nutrient channel spacing and revisiting TGF-beta in large engineered cartilage constructs. J Biomech 49:2089–2094

Cleary MA, van Osch GJ, Brama PA, Hellingman CA, Narcisi R (2015) FGF, TGF beta and Wnt crosstalk: embryonic to in vitro cartilage development from mesenchymal stem cells. J Tissue Eng Regen Med 9(4):332–342

Cole BJ, Farr J, Winalski CS, Hosea T, Richmond J, Mandelbaum B, De Deyne PG (2011) Outcomes after a single-stage procedure for cell-based cartilage repair: a prospective clinical safety trial with 2-year follow-up. Am J Sports Med 39:1170–1179

Concaro S, Concaro C (2016) Cell transplantation for cartilage repair. An update. Aspetar Sports Med J 5:252–255

Conde J, Otero M, Scotece M, Abella V, Lopez V, Pino J, Gomez R, Lago F, Goldring MB, Gualillo O (2016) E74-like factor 3 and nuclear factor-kappaB regulate lipocalin-2 expression in chondrocytes. J Physiol 594:6133–6146

Correa D, Somoza RA, Lin P, Greenberg S, Rom E, Duesler L, Welter JF, Yayon A, Caplan AI (2015) Sequential exposure to fibroblast growth factors (FGF) 2, 9 and 18 enhances hMSC chondrogenic differentiation. Osteoarthr Cartil 23:443–453

Correro-Shahgaldian MR, Ghayor C, Spencer ND, Weber FE, Gallo LM (2014) A model system of the dynamic loading occurring in synovial joints: the biological effect of plowing on pristine cartilage. Cells Tissues Organs 199:364–372

Crawford DC, DeBerardino TM, Williams RJ 3rd (2012) NeoCart, an autologous cartilage tissue implant, compared with microfracture for treatment of distal femoral cartilage lesions: an FDA phase-II prospective, randomized clinical trial after two years. J Bone Joint Surg Am 94:979–989

Cui X, Hasegawa A, Lotz M, D'Lima D (2012) Structured three-dimensional co-culture of mesenchymal stem cells with meniscus cells promotes meniscal phenotype without hypertrophy. Biotechnol Bioeng 109(9):2369–2380

Darling EM, Hu JC, Athanasiou KA (2004) Zonal and topographical differences in articular cartilage gene expression. J Orthop Res 22:1182–1187

Da Silva Meirelles L, Caplan AI, Nardi NB (2008) In search of the in vivo identity of mesenchymal stem cells. Stem Cells 26:2287–2299

Dashtdar H, Rothan HA, Tay T, Ahmad RE, Ali R, Tay LX, Chong PP, Kamarul T (2011) A preliminary study comparing the use of allogenic chondrogenic pre-differentiated and undifferentiated mesenchymal stem cells for the repair of full thickness articular cartilage defects in rabbits. J Orthop Res 29:1336–1342

De Bari C, Dell'Accio F, Luyten FP (2001) Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. Arthritis Rheum 44:85–95

De Boer J, Wang HJ, van Blitterswijk C (2004) Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. Tissue Eng 10:393–401

Decker RS, Koyama E, Pacifici M (2014) Genesis and morphogenesis of limb synovial joints and articular cartilage. Matrix Biol 39:5–10

Decker RS, Koyama E, Pacifici M (2015) Articular cartilage: structural and developmental intricacies and questions. Curr Osteoporos Rep 13:407–414

Dell'Accio F, De BC, Luyten FP (2001) Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. Arthritis Rheum 44:1608–1619

DeLise AM, Fischer L, Tuan RS (2000) Cellular interactions and signaling in cartilage development. Osteoarthr Cartil 8:309–334

Deshmukh K, Kline WH (1976) Characterization of collagen and its precursors synthesized by rabbit-articular-cartilage cells in various culture systems. Eur J Biochem 69:117–123

De Vries-van Melle ML, Narcisi R, Kops N, Koevoet WJ, Bos PK, Murphy JM, Verhaar JA, van der Kraan PM, van Osch GJ (2014) Chondrogenesis of mesenchymal stem cells in an osteochondral environment is mediated by the subchondral bone. Tissue Eng Part A 20:23–33

De Windt TS, Vonk LA, Slaper-Cortenbach IC, van den Broek MP, Nizak R, van Rijen MH, de Weger RA, Dhert WJ, Saris DB (2016) Allogeneic mesenchymal stem cells stimulate cartilage regeneration and are safe for single-stage cartilage repair in humans upon mixture with recycled autologous Chondrons. Stem Cells 35:256–264

Diaz-Gomez L, Alvarez-Lorenzo C, Concheiro A, Silva M, Dominguez F, Sheikh FA, Cantu T, Desai R, Garcia VL, Macossay J (2014) Biodegradable electrospun nanofibers coated with platelet-rich plasma for cell adhesion and proliferation. Mater Sci Eng C Mater Biol Appl 40:180–188

Diaz-Flores L, Gutierrez R, Gonzalez P, Varela H (1991) Inducible perivascular cells contribute to the neochondrogenesis in grafted perichondrium. Anat Rec 229:1–8

Diekman BO, Wu C-L, Louer CR, Furman BD, Huebner JL, Kraus VB, Olson SA, Guilak F (2013) Intra-articular delivery of purified mesenchymal stem cells from C57BL/6 or MRL/MpJ superhealer mice prevents posttraumatic arthritis. Cell Transplant 22:1395–1408

Do Amaral RJ, Pedrosa CS, Kochem MC, Silva KR, Aniceto M, Claudio-da-Silva C, Borojevic R, Baptista LS (2012) Isolation of human nasoseptal chondrogenic cells: a promise for cartilage engineering. Stem Cell Res 8:292–299

Dobratz EJ, Kim SW, Voglewede A, Park SS (2009) Injectable cartilage: using alginate and human chondrocytes. Arch Facial Plast Surg 11:40–47

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

Doucet C, Ernou I, Zhang Y, Llense J-R, Begot L, Holy X, Lataillade J-J (2005) Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. J Cell Physiol 205:228–236

Dozin B, Malpeli M, Cancedda R, Bruzzi P, Calcagno S, Molfetta L, Priano F, Kon E, Marcacci M (2005) Comparative evaluation of autologous chondrocyte implantation and mosaicplasty: a multicentered randomized clinical trial. Clin J Sport Med 15:220–226

Elsaesser AF, Bermueller C, Schwarz S, Koerber L, Breiter R, Rotter N (2014) In vitro cytotoxicity and in vivo effects of a decellularized xenogeneic collagen scaffold in nasal cartilage repair. Tissue Eng Part A 20:1668–1678

Elsaesser AF, Schwarz S, Joos H, Koerber L, Brenner RE, Rotter N (2016) Characterization of a migrative subpopulation of adult human nasoseptal chondrocytes with progenitor cell features and their potential for in vivo cartilage regeneration strategies. Cell Biosci 6:11. https://doi.org/10.1186/s13578-016-0078-6. eCollection@2016.:11-0078

El Sayed K, Haisch A, John T, Marzahn U, Lohan A, Müller RD, Kohl B, Ertel W, Stoelzel K, Schulze-Tanzil G (2010) Heterotopic autologous chondrocyte transplantation – a realistic approach to support articular cartilage repair? Tissue Eng Part B Rev 16:603–616

Engen CN, Engebretsen L, Årøen A (2010) Knee cartilage defect patients enrolled in randomized controlled trials are not representative of patients in Orthopedic practice. Cartilage 1:312–319

Elvidge J, Bullement A, Hatswell AJ (2016) Cost effectiveness of characterised chondrocyte implantation for treatment of cartilage defects of the knee in the UK. PharmacoEconomics 34:1145–1159

Farhadi J, Fulco I, Miot S, Wirz D, Haug M, Dickinson SC, Hollander AP, Daniels AU, Pierer G, Heberer M, Martin I (2006) Precultivation of engineered human nasal cartilage enhances the mechanical properties relevant for use in facial reconstructive surgery. Ann Surg 244:978–985

Faqeh Al H, Nor Hamdan BMY, Chen H-C, Aminuddin BS, Ruszymah BHI (2012) The potential of intra-articular injection of chondrogenic-induced bone marrow stem cells to retard the progression of osteoarthritis in a sheep model. Exp Gerontol 47:458–464

Fekete N, Rojewski MT, Lotfi R, Schrezenmeier H (2014) Essential components for ex vivo proliferation of mesenchymal stromal cells. Tissue Eng Part C Methods 20:129–139

Ferlin KM, Prendergast ME, Miller ML, Kaplan DS, Fisher JP (2016) Influence of 3D printed porous architecture on mesenchymal stem cell enrichment and differentiation. Acta Biomater 32:161–169

Fierro F, Illmer T, Jing D, Schleyer E, Ehninger G, Boxberger S, Bornhauser M (2007) Inhibition of platelet-derived growth factor receptorbeta by imatinib mesylate suppresses proliferation and alters differentiation of human mesenchymal stem cells in vitro. Cell Prolif 40:355–366

Finger F, Schorle C, Soder S, Zien A, Goldring MB, Aigner T (2004) Phenotypic characterization of human chondrocyte cell line C-20/A4: a comparison between monolayer and alginate suspension culture. Cells Tissues Organs 178(2):65–77

Finger F, Schorle C, Zien A, Gebhard P, Goldring MB, Aigner T (2003) Molecular phenotyping of human chondrocyte cell lines T/C-28a2, T/C-28a4, and C-28/I2. Arthritis Rheum 48 (12):3395–3403

Francis-West PH, Parish J, Lee K, Archer CW (1999a) BMP/GDF-signalling interactions during synovial joint development. Cell Tissue Res 296(1):111–9

Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten FP, Archer CW (1999b) Mechanisms of GDF-5 action during skeletal development. Development 126(6):1305–15

Freymann U, Endres M, Neumann K, Scholman HJ, Morawietz L, Kaps C (2012) Expanded human meniscus-derived cells in 3-D polymer-hyaluronan scaffolds for meniscus repair. Acta Biomater 8:677–685

Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 2:83–92

Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393–403

Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274

Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16:381–390

Frisbie DD, Kisiday JD, Kawcak CE, Werpy NM, McIlwraith CW (2009) Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. J Orthop Res 27:1675–1680

Fulco I, Miot S, Haug MD, Barbero A, Wixmerten A, Feliciano S, Wolf F, Jundt G, Marsano A, Farhadi J, Heberer M, Jakob M, Schaefer DJ, Martin I (2014) Engineered autologous cartilage tissue for nasal reconstruction after tumour resection: an observational first-in-human trial. Lancet 384:337–346

Furumatsu T, Kanazawa T, Yokoyama Y, Abe N, Ozaki T (2011) Inner meniscus cells maintain higher chondrogenic phenotype compared with outer meniscus cells. Connect Tissue Res 52:459–465

Gadjanski I, Spiller K, Vunjak-Novakovic G (2012) Time-dependent processes in stem cell-based tissue engineering of articular cartilage. Stem Cell Rev 8:863–881

Gardner OF, Fahy N, Alini M, Stoddart MJ (2016) Joint mimicking mechanical load activates TGFbeta1 in fibrin-poly(ester-urethane) scaffolds seeded with mesenchymal stem cells. J Tissue Eng Regen Med 11:2663–2666

Gebauer M, Saas J, Sohler F, Haag J, Soder S, Pieper M, Bartnik E, Beninga J, Zimmer R, Aigner T (2005) Comparison of the chondrosarcoma cell line SW1353 with primary human adult articular chondrocytes with regard to their gene expression profile and reactivity to IL-1beta. Osteoarthr Cartil 13(8):697–708

Ghazanfari R, Li H, Zacharaki D, Lim HC, Scheding S (2016) Human non-hematopoietic CD271pos/CD140alow/neg bone marrow stroma cells Fulfill stringent stem cell criteria in serial transplantations. Stem Cells Dev. https://doi.org/10.1089/scd.2016.0169

Giannini S, Buda R, Vannini F, Cavallo M, Grigolo B (2009) One-step bone marrow-derived cell transplantation in talar osteochondral lesions. Clin Orthop Relat Res 467:3307–3320

Gionti E, Pontarelli G, Cancedda R (1985) Avian myelocytomatosis virus immortalizes differentiated quail chondrocytes. Proc Natl Acad Sci U S A 82:2756–2760

Glenister TW (1976) An embryological view of cartilage. J Anat 122:323–330

Glowacki J, Trepman E, Folkman J (1983) Cell shape and phenotypic expression in chondrocytes. Proc Soc Exp Biol Med 172:93–98

Goldring MB (2004a) Culture of immortalized chondrocytes and their use as models of chondrocyte function. Methods Mol Med 100:37–52

Goldring MB (2004b) Immortalization of human articular chondrocytes for generation of stable, differentiated cell lines. Methods Mol Med 100:23–36

Goldring MB, Tsuchimochi K, Ijiri K (2006) The control of chondrogenesis. J Cell Biochem 97:33–44

Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, Arbiser JL, Apperley JF (1994) Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. J Clin Invest 94:2307–2316

Goldring MB, Sandell LJ, Stephenson ML, Krane SM (1986) Immune interferon suppresses levels of procollagen mRNA and type II collagen synthesis in cultured human articular and costal chondrocytes. J Biol Chem 261(19):9049–9055

Gooding CR, Bartlett W, Bentley G, Skinner JA, Carrington R, Flanagan A (2006) A prospective, randomised study comparing two techniques of autologous chondrocyte implantation for osteochondral defects in the knee: periosteum covered versus type I/III collagen covered. Knee 13:203–210

Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471(7336):63–67

Grall FT, Prall WC, Wei W, Gu X, Cho JY, Choy BK, Zerbini LF, Inan MS, Goldring SR, Gravallese EM, Goldring MB, Oettgen P, Libermann TA (2005) The Ets transcription factor ESE-1 mediates induction of the COX-2 gene by LPS in monocytes. FEBS J 272(7):1676–1687

Grayson WL, Bhumiratana S, Grace Chao PH, Hung CT, Vunjak-Novakovic G (2010) Spatial regulation of human mesenchymal stem cell differentiation in engineered osteochondral constructs: effects of pre-differentiation, soluble factors and medium perfusion. Osteoarthr Cartil 18:714–723

Greco KV, Iqbal AJ, Rattazzi L, Nalesso G, Moradi-Bidhendi N, Moore AR, Goldring MB, Dell'accio F, Perretti M (2011) High density micromass cultures of a human chondrocyte cell line: a reliable assay system to reveal the modulatory functions of pharmacological agents. Biochem Pharmacol 82:1919–1929

Greco KV, Nalesso G, Kaneva MK, Sherwood J, Iqbal AJ, Moradi-Bidhendi N, Dell'Accio F, Perretti M (2014) Analyses on the mechanisms that underlie the chondroprotective properties of calcitonin. Biochem Pharmacol 91:348–358

Grigolo B, Roseti L, Neri S, Gobbi P, Jensen P, Major EO, Facchini A (2002) Human articular chondrocytes immortalized by HPV-16 E6 and E7 genes: maintenance of differentiated phenotype under defined culture conditions. Osteoarthr Cartil 10(11):879–889

Grigoriadis AE, Heersche JN, Aubin JE (1988) Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol 106(6):2139–2151

Guilak F, Butler DL, Goldstein SA, Baaijens FP (2014) Biomechanics and mechanobiology in functional tissue engineering. J Biomech 47(9):1933–1940

Gupta PK, Das AK, Chullikana A, Majumdar AS (2012) Mesenchymal stem cells for cartilage repair in osteoarthritis. Stem Cell Res Ther 3:25

Haisch A, Klaring S, Groger A, Gebert C, Sittinger M (2002) A tissue-engineering model for the manufacture of auricular-shaped cartilage implants. Eur Arch Otorhinolaryngol 259:316–321

Hall BK, Miyake T (2000) All for one and one for all: condensations and the initiation of skeletal development. Bioessays 22:138–147

Hamamura K, Goldring MB, Yokota H (2009) Involvement of p38 MAPK in regulation of MMP13 mRNA in chondrocytes in response to surviving stress to endoplasmic reticulum. Arch Oral Biol 54:279–286

Handorf AM, Li WJ (2011) Fibroblast growth factor-2 primes human mesenchymal stem cells for enhanced chondrogenesis. PLoS One 6:e22887

Haraguchi Y, Matsuura K, Shimizu T, Yamato M, Okano T (2015) Simple suspension culture system of human iPS cells maintaining their pluripotency for cardiac cell sheet engineering. J Tissue Eng Regen Med 9(12):1363–1375

Hashimoto K, Otero M, Imagawa K, de Andres MC, Coico JM, Roach HI, Oreffo RO, Marcu KB, Goldring MB (2013) Regulated transcription of human matrix metalloproteinase 13 (MMP13) and interleukin-1beta (IL1B) genes in chondrocytes depends on methylation of specific proximal promoter CpG sites. J Biol Chem 288:10061–10072

Healy ZR, Lee NH, Gao X, Goldring MB, Talalay P, Kensler TW, Konstantopoulos K (2005) Divergent responses of chondrocytes and endothelial cells to shear stress: cross-talk among COX-2, the phase 2 response, and apoptosis. Proc Natl Acad Sci U S A 102(39):14010–14015

Heinemeier KM, Schjerling P, Heinemeier J, Møller MB, Krogsgaard MR, Grum-Schwensen T, Petersen MM, Kjaer M (2016) Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. Sci Transl Med 8(346):346ra9

Hellingman CA, Verwiel ET, Slagt I, Koevoet W, Poublon RM, Nolst-Trenité GJ, Baatenburg de Jong RJ, Jahr H, van Osch GJ (2011a) Differences in cartilage-forming capacity of expanded human chondrocytes from ear and nose and their gene expression profiles. Cell Transplant 20:925–940

Hellingman CA, Davidson ENB, Koevoet W, Vitters EL, van den Berg WB, van Osch GJVM, van der Kraan PM (2011b) Smad signaling determines chondrogenic differentiation of bone-

marrow-derived mesenchymal stem cells: inhibition of Smad1/5/8P prevents terminal differentiation and calcification. Tissue Eng Part A 17:1157–1167

Helms JA, Cordero D, Tapadia MD (2005) New insights into craniofacial morphogenesis. Development 132:851–861

Hermida-Gómez T, Fuentes-Boquete I, Gimeno-Longas MJ, Muiños-López E, Díaz-Prado S, de Toro FJ, Blanco FJ (2011) Bone marrow cells immunomagnetically selected for CD271+ antigen promote in vitro the repair of articular cartilage defects. Tissue Eng Part A 17:1169–1179

Hohman MH, Lindsay RW, Pomerantseva I, Bichara DA, Zhao X, Johnson M, Kulig KM, Sundback CA, Randolph MA, Vacanti JP, Cheney ML, Hadlock TA (2014) Ovine model for auricular reconstruction: porous polyethylene implants. Ann Otol Rhinol Laryngol 123:135–140

Holden PK, Liaw LH, Wong BJ (2008) Human nasal cartilage ultrastructure: characteristics and comparison using scanning electron microscopy. Laryngoscope 118:1153–1156

Holder N (1977) An experimental investigation into the early development of the chick elbow joint. J Embryol Exp Morphol 39:115–127

Homicz MR, Chia SH, Schumacher BL, Masuda K, Thonar EJ, Sah RL, Watson D (2003) Human septal chondrocyte redifferentiation in alginate, polyglycolic acid scaffold, and monolayer culture. Laryngoscope 113:25–32

Homicz MR, Schumacher BL, Sah RL, Watson D (2002) Effects of serial expansion of septal chondrocytes on tissue-engineered neocartilage composition. Otolaryngol Head Neck Surg 127:398–408

Horas U, Pelinkovic D, Herr G, Aigner T, Schnettler R (2003) Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial. J Bone Joint Surg Am 85-A:185–192

Horie M, Choi H, Lee RH, Reger RL, Ylostalo J, Muneta T, Sekiya I, Prockop DJ (2012) Intra-articular injection of human mesenchymal stem cells (MSCs) promote rat meniscal regeneration by being activated to express Indian hedgehog that enhances expression of type II collagen. Osteoarthr Cartil 20:1197–1207

Horwitz AL, Dorfman A (1970) The growth of cartilage cells in soft agar and liquid suspension. J Cell Biol 45:434–438

Huang BJ, Hu JC, Athanasiou KA (2016) Cell-based tissue engineering strategies used in the clinical repair of articular cartilage. Biomaterials 98:1–22

Huurne ter M, Schelbergen R, Blattes R, Blom A, de Munter W, Grevers LC, Jeanson J, Noël D, Casteilla L, Jorgensen C, van den Berg W, van Lent PLEM (2012) Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. Arthritis Rheum 64:3604–3613

Hsu SH, Chang SH, Yen HJ, Whu SW, Tsai CL, Chen DC (2006) Evaluation of biodegradable polyesters modified by type II collagen and Arg-Gly-asp as tissue engineering scaffolding materials for cartilage regeneration. Artif Organs 30:42–55

Hubka KM, Dahlin RL, Meretoja VV, Kasper FK, Mikos AG (2014) Enhancing chondrogenic phenotype for cartilage tissue engineering: monoculture and coculture of articular chondrocytes and mesenchymal stem cells. Tissue Eng Part B Rev 20:641–654

Hyde G, Boot-Handford RP, Wallis GA (2008) Col2a1 lineage tracing reveals that the meniscus of the knee joint has a complex cellular origin. J Anat 213:531–538

Hyde G, Dover S, Aszodi A, Wallis GA, Boot-Handford RP (2007) Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. Dev Biol 304:825–833

Ijiri K, Zerbini LF, Peng H, Correa RG, Lu B, Walsh N, Zhao Y, Taniguchi N, Huang XL, Otu H, Wang H, Fei Wang J, Komiya S, Ducy P, Rahman MU, Flavell RA, Libermann TA, Gravallese EM, Oettgen P, Goldring MB (2005) A novel role for GADD45b as a mediator of MMP-13 gene expression during chondrocyte terminal differentiation. J Biol Chem 280(46):38544–38555

Im HJ, Pacione C, Chubinskaya S, Van Wijnen AJ, Sun Y, Loeser RF (2003) Inhibitory effects of insulin-like growth factor-1 and osteogenic protein-1 on fibronectin fragment- and interleukin-1beta-stimulated matrix metalloproteinase-13 expression in human chondrocytes. J Biol Chem 278:25386–25394

Ishizeki K, Shinagawa T, Nawa T (2003) Origin-associated features of chondrocytes in mouse Meckel's cartilage and costal cartilage: an in vitro study. Ann Anat 185:403–410

Isogai N, Morotomi T, Hayakawa S, Munakata H, Tabata Y, Ikada Y, Kamiishi H (2005) Combined chondrocyte-copolymer implantation with slow release of basic fibroblast growth factor for tissue engineering an auricular cartilage construct. J Biomed Mater Res A 74:408–418

Isogai N, Kusuhara H, Ikada Y, Ohtani H, Jacquet R, Hillyer J, Lowder E, Landis WJ (2006) Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. Tissue Eng 12:691–703

Ito T, Sawada R, Fujiwara Y, Seyama Y, Tsuchiya T (2007) FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF-beta2. Biochem Biophys Res Commun 359:108–114

Iwamoto M, Higuchi Y, Enomoto-Iwamoto M, Kurisu K, Koyama E, Yeh H, Rosenbloom J, Pacifici M (2001) The role of ERG (ets related gene) in cartilage development. Osteoarthr Cartil 9:S41–S47

Iwamoto M, Koyama E, Enomoto-Iwamoto M, Pacifici M (2005) The balancing act of transcription factors C-1-1 and Runx2 in articular cartilage development. Biochem Biophys Res Commun 328:777–182

Jayasuriya CT, Goldring MB, Terek R, Chen Q (2012) Matrilin-3 induction of IL-1 receptor antagonist is required for up-regulating collagen II and aggrecan and down-regulating ADAMTS-5 gene expression. Arthritis Res Ther 14:R197

Jenner F, IJpma A, Cleary M, Heijsman D, Narcisi R, van der Spek PJ, Kremer A, van Weeren R, Brama P, van Osch GJ (2014) Differential gene expression of the intermediate and outer interzone layers of developing articular cartilage in murine embryos. Stem Cells Dev 23:1883–1898

Jian-Wei X, Randolph MA, Peretti GM, Nazzal JA, Roses RE, Morse KR, Yaremchuk MJ (2005) Producing a flexible tissue-engineered cartilage framework using expanded poly-tetrafluoroethylene membrane as a pseudoperichondrium. Plast Reconstr Surg 116:577–589

Jian H, Shen X, Liu I, Semenov M, He X, Wang X-F (2006) Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes Dev 20:666–674

Joana Frobel, Hatim Hemeda, Michael Lenz, Giulio Abagnale, Sylvia Joussen, Bernd Denecke, Tomo Šarić, Martin Zenke, Wolfgang Wagner, (2014) Epigenetic Rejuvenation of Mesenchymal Stromal Cells Derived from Induced Pluripotent Stem Cells. Stem Cell Reports 3 (3):414–422

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272

Johnstone B, Barthel T, Yoo J (2003) In vitro chondrogenesis with mammalian progenitor cells. In: Rosier RV, Evans CH (eds) Molecular biology of orthopaedics. American Academy of Orthopaedic Surgeons, Rosemont, pp 273–287

Judson RL, Babiarz JE, Venere M, Blelloch R (2009) Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol 27 (5):459–61

Kahn J, Shwartz Y, Blitz E, Krief S, Sharir A, Breitel DA, Rattenbach R, Relaix F, Maire P, Rountree RB, Kingsley DM, Zelzer E (2009) Muscle contraction is necessary to maintain joint progenitor cell fate. Dev Cell 16:734–743

Kaltz N, Ringe J, Holzwarth C, Charbord P, Niemeyer M, Jacobs VR, Peschel C, Häupl T, Oostendorp RAJ (2010) Novel markers of mesenchymal stem cells defined by genome-wide gene expression analysis of stromal cells from different sources. Exp Cell Res 316:2609–2617

Kamil SH, Vacanti MP, Vacanti CA, Eavey RD (2004a) Microtia chondrocytes as a donor source for tissue-engineered cartilage. Laryngoscope 114:2187–2190

Kamil SH, Vacanti MP, Aminuddin BS, Jackson MJ, Vacanti CA, Eavey RD (2004b) Tissue engineering of a human sized and shaped auricle using a mold. Laryngoscope 114:867–870
Katsutsugu Umeda, Jiangang Zhao, Paul Simmons, Edouard Stanley, Andrew Elefanty, Naoki Nakayama, (2012) Human chondrogenic paraxial mesoderm, directed specification and prospective isolation from pluripotent stem cells. Scientific Reports 2 (1)
Kazutoshi Takahashi, Shinya Yamanaka, (2006) Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126 (4):663–676
Kazutoshi Takahashi, Shinya Yamanaka, (2016) A decade of transcription factor-mediated reprogramming to pluripotency. Nature Reviews Molecular Cell Biology 17 (3):183–193
Khozoee B, Mafi P, Mafi R, Khan W (2016) Mechanical stimulation protocols of human derived cells in articular cartilage tissue engineering - a systematic review. Curr Stem Cell Res Ther 12:260–270
Kilian KA, Bugarija B, Lahn BT, Mrksich M (2010) Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci U S A 107:4872–4877
Kim SW, Dobratz EJ, Ballert JA, Voglewede AT, Park SS (2009) Subcutaneous implants coated with tissue-engineered cartilage. Laryngoscope 119:62–66
Kirsch T, Swoboda B, Nah H (2000) Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage. Osteoarthr Cartil 8:294–302
Kitagawa F, Takei S, Imaizumi T, Tabata Y (2013) Chondrogenic differentiation of immortalized human mesenchymal stem cells on zirconia microwell substrata. Tissue Eng Part C Methods 19:438–448
Klein TJ, Malda J, Sah RL, Hutmacher DW (2009) Tissue engineering of articular cartilage with biomimetic zones. Tissue Eng Part B Rev 15:143–157
Klockars T, Rautio J (2009) Embryology and epidemiology of microtia. Facial Plast Surg 25:145–148
Kock L, van Donkelaar CC, Ito K (2012) Tissue engineering of functional articular cartilage: the current status. Cell Tissue Res 347:613–627
Koga H, Muneta T, Nagase T, Nimura A, Y-J J, Mochizuki T, Sekiya I (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 333:207–215
Koh Y-G, Choi Y-J (2012) Infrapatellar fat pad-derived mesenchymal stem cell therapy for knee osteoarthritis. Knee 19:902–907
Koji Yokoyama, Makoto Ikeya, Katsutsugu Umeda, Hirotsugu Oda, Seishiro Nodomi, Akira Nasu, Yoshihisa Matsumoto, Kazushi Izawa, Kazuhiko Horigome, Toshimasa Kusaka, Takayuki Tanaka, Megumu K. Saito, Takahiro Yasumi, Ryuta Nishikomori, Osamu Ohara, Naoki Nakayama, Tatsutoshi Nakahata, Toshio Heike, Junya Toguchida, (2015) Enhanced Chondrogenesis of Induced Pluripotent Stem Cells From Patients With Neonatal-Onset Multisystem Inflammatory Disease Occurs via the Caspase 1-Independent cAMP/Protein Kinase A/CREB Pathway. Arthritis & Rheumatology 67 (1):302–314
Kokenyesi R, Tan L, Robbins JR, Goldring MB (2000) Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys 383(1):79–90
Kolettas E, Buluwela L, Bayliss MT, Muir HI (1995) Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. J Cell Sci 108(Pt 5):1991–1999
Knutsen G, Drogset JO, Engebretsen L, Grøntvedt T, Isaksen V, Ludvigsen TC, Roberts S, Solheim E, Strand T, Johansen O (2007) A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. J Bone Joint Surg Am 89:2105–2112
Kon E, Roffi A, Filardo G, Tesei G, Marcacci M (2015) Scaffold-based cartilage treatments: with or without cells? A systematic review of preclinical and clinical evidence. Arthroscopy 31:767–775
Koyama E, Shibukawa Y, Nagayama M, Sugito H, Young B, Yuasa T, Okabe T, Ochiai T, Kamiya N, Rountree RB, Kingsley DM, Iwamoto M, Enomoto-Iwamoto M, Pacifici M (2008) A distinct

cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. Dev Biol 316:62–73

Kremer A, Ribitsch I, Reboredo J, Dürr J, Egerbacher M, Jenner F, Walles H (2017) Three-dimensional Coculture of meniscal cells and mesenchymal stem cells in collagen type I hydrogel on a small intestinal matrix-a pilot study toward equine meniscus tissue engineering. Tissue Eng Part A 23(9–10):390–402

Kuroda R, Ishida K, Matsumoto T, Akisue T, Fujioka H, Mizuno K, Ohgushi H, Wakitani S, Kurosaka M (2007) Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. Osteoarthr Cartil 15:226–231

Kusuhara H, Isogai N, Enjo M, Otani H, Ikada Y, Jacquet R et al (2009) Tissue engineering a model for the human ear: assessment of size, shape, morphology, and gene expression following seeding of different chondrocytes. Wound Repair Regen 17:136–146

Lamo-Espinosa JM, Mora G, Blanco JF, Granero-Moltó F, Nuñez-Córdoba JM, Sánchez-Echenique C, Bondía JM, Aquerreta JD, Andreu EJ, Ornilla E, Villarón EM, Valentí-Azcárate A, Sánchez-Guijo F, del Cañizo MC, Valentí-Nin JR, Prósper F (2016) Intra-articular injection of two different doses of autologous bone marrow mesenchymal stem cells versus hyaluronic acid in the treatment of knee osteoarthritis: multicenter randomized controlled clinical trial (phase I/II). J Transl Med 14:246

Lamplot JD, Liu B, Yin L, Zhang W, Wang Z, Luther G, Wagner E, Li R, Nan G, Shui W, Yan Z, Rames R, Deng F, Zhang H, Liao Z, Liu W, Zhang J, Zhang Z, Zhang Q, Ye J, Deng Y, Qiao M, Haydon RC, Luu HH, Angeles J, Shi LL, He TC, Ho SH (2015) Reversibly immortalized mouse articular chondrocytes acquire long-term proliferative capability while retaining Chondrogenic phenotype. Cell Transplant 24:1053–1066

Larribere L, Wu H, Novak D, Galach M, Bernhardt M, Orouji E, Weina K, Knappe N, Sachpekidis C, Umansky L, Beckhove P, Umansky V, De Schepper S, Kaufmann D, Ballotti R, Bertolotto C, Utikal J (2015) NF1 loss induces senescence during human melanocyte differentiation in an iPSC-based model. Pigment Cell Melanoma Res 28(4):407–416

Laurent LC, Ulitsky I, Slavin I, Tran H, Schork A, Morey R, Lynch C, Harness JV, Lee S, Barrero MJ, Ku S, Martynova M, Semechkin R, Galat V, Gottesfeld J, Izpisua Belmonte JC, Murry C, Keirstead HS, Park HS, Schmidt U, Laslett AL, Muller FJ, Nievergelt CM, Shamir R, Loring JF (2011) Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. Cell Stem Cell 8 (1):106–118

Lee TS, Lim SY, Pyon JK, Mun GH, Bang SI, KS O (2010) Secondary revisions due to unfavourable results after microtia reconstruction. J Plast Reconstr Aesthet Surg 63:940–946

Lee J, Abdeen AA, Zhang D, Kilian KA (2013) Directing stem cell fate on hydrogel substrates by controlling cell geometry, matrix mechanics and adhesion ligand composition. Biomaterials 34:8140–8148

Lee KBL, Hui JHP, Song IC, Ardany L, Lee EH (2007) Injectable mesenchymal stem cell therapy for large cartilage defects–a porcine model. Stem Cells 25:2964–2971

Lefebvre V, Smits P (2005) Transcriptional control of chondrocyte fate and differentiation. Birth Defect Res C 75:200–212

Lefebvre V, Garofalo S, de Crombrugghe B (1995) Type X collagen gene expression in mouse chondrocytes immortalized by a temperature-sensitive simian virus 40 large tumor antigen. J Cell Biol 128(1–2):239–245

Leijten J, Georgi N, Moreira Teixeira L, van Blitterswijk CA, Post JN, Karperien M (2014) Metabolic programming of mesenchymal stromal cells by oxygen tension directs chondrogenic cell fate. Proc Natl Acad Sci U S A 111:13954–13959

Leucht P, Kim JB, Amasha R, James AW, Girod S, Helms JA (2008) Embryonic origin and Hox status determine progenitor cell fate during adult bone regeneration. Development 135:2845–2854

Li Y, Tew SR, Russell AM, Gonzalez KR, Hardingham TE, Hawkins RE (2004) Transduction of passaged human articular chondrocytes with adenoviral, retroviral, and lentiviral vectors and the effects of enhanced expression of SOX9. Tissue Eng 10(3–4):575–584

Liese J, Marzahn U, El SK, Pruss A, Haisch A, Stoelzel K (2013) Cartilage tissue engineering of nasal septal chondrocyte-macroaggregates in human demineralized bone matrix. Cell Tissue Bank 14:255–266

Lim HC, Bae JH, Song SH, Park YE, Kim SJ (2012) Current treatments of isolated articular cartilage lesions of the knee achieve similar outcomes. Clin Orthop Relat Res 470:2261–2267

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

Lolli A, Narcisi R, Lambertini E, Penolazzi L, Angelozzi M, Kops N, Gasparini S, van Osch GJVM, Piva R (2016) Silencing of Antichondrogenic MicroRNA-221 in human mesenchymal stem cells promotes cartilage repair in vivo. Stem Cells 34:1801–1811

Locklin RM, Riggs BL, Hicok KC, Horton HF, Byrne MC, Khosla S (2001) Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology. J Bone Miner Res 16:2192–2204

Loeser RF, Sadiev S, Tan L, Goldring MB (2000) Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthr Cartil 8(2):96–105

Lotz M, Loeser RF (2012) Effects of aging on articular cartilage homeostasis. Bone 51(2):241–248

Luan Y, Kong L, Howell DR, Ilalov K, Fajardo M, Bai XH, Di Cesare PE, Goldring MB, Abramson SB, Liu CJ (2008) Inhibition of ADAMTS-7 and ADAMTS-12 degradation of cartilage oligomeric matrix protein by alpha-2-macroglobulin. Osteoarthr Cartil 16:1413–1420

Madry H, Orth P, Cucchiarini M (2011) Gene therapy for cartilage repair. Cartilage 2(3):201–225

Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, Carmeliet G, Kronenberg HM (2010) Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev Cell 19:329–344

Makris EA, Hadidi P, Athanasiou KA (2011) The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. Biomaterials 32 (30):7411–7431

Malda J, Kreijveld E, Temenoff JS, van Blitterswijk CA, Riesle J (2003) Expansion of human nasal chondrocytes on macroporous microcarriers enhances redifferentiation. Biomaterials 24:5153–5161

Mallein-Gerin F, Ruggiero F, Quinn TM, Bard F, Grodzinsky AJ, Olsen BR, van der Rest M (1995) Analysis of collagen synthesis and assembly in culture by immortalized mouse chondrocytes in the presence or absence of alpha 1(IX) collagen chains. Exp Cell Res 219:257–265

Matthies N-F, Mulet-Sierra A, Jomha NM, Adesida AB (2012) Matrix formation is enhanced in co-cultures of human meniscus cells with bone marrow stromal cells. J Tissue Eng Regen Med 7 (12):965–973

Mauck RL, Martinez-Diaz GJ, Yuan X, Tuan RS (2007) Regional multilineage differentiation potential of meniscal fibrochondrocytes: implications for meniscus repair. Anat Rec (Hoboken) 290:48–58

Marquass B, Schulz R, Hepp P, Zscharnack M, Aigner T, Schmidt S, Stein F, Richter R, Osterhoff G, Aust G, Josten C, Bader A (2011) Matrix-associated implantation of predifferentiated mesenchymal stem cells versus articular chondrocytes: in vivo results of cartilage repair after 1 year. Am J Sports Med 39:1401–1412

Marsano A, Medeiros da Cunha CM, Ghanaati S, Gueven S, Centola M, Tsaryk R, Barbeck M, Stuedle C, Barbero A, Helmrich U, Schaeren S, Kirkpatrick JC, Banfi A, Martin I (2016) Spontaneous in vivo Chondrogenesis of bone marrow-derived mesenchymal progenitor cells by blocking vascular endothelial growth factor Signaling. Stem Cells Transl Med sctm 5:1730–1738

Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R (1997) Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 138:4456–4462

Mataga N, Tamura M, Yanai N, Shinomura T, Kimata K, Obinata M, Noda M (1996) Establishment of a novel chondrocyte-like cell line derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen. J Bone Min Res 11:1646–1654

McIntyre DC, Rakshit S, Yallowitz AR, Loken L, Jeannotte L, Capecchi MR, Wellik DM (2007) Hox patterning of the vertebrate rib cage. Development 134:2981–2989

McIlwraith CW, Frisbie DD, Rodkey WG, Kisiday JD, Werpy NM, Kawcak CE, Steadman JR (2011) Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects. Arthroscopy 27:1552–1561

Mendelson A, Frenette PS (2014) Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nat Med 20:833–846

Mihaela Crisan, Solomon Yap, Louis Casteilla, Chien-Wen Chen, Mirko Corselli, Tea Soon Park, Gabriella Andriolo, Bin Sun, Bo Zheng, Li Zhang, Cyrille Norotte, Pang-Ning Teng, Jeremy Traas, Rebecca Schugar, Bridget M. Deasy, Stephen Badylak, Hans-Jörg Bűhring, Jean-Paul Giacobino, Lorenza Lazzari, Johnny Huard, Bruno Péault, (2008) A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell 3 (3):301–313

Miller EJ (1976) Biochemical characteristics and biological significance of the genetically-distinct collagens. Mol Cell Biochem 13:165–192

Minoru Okada, Shiro Ikegawa, Miho Morioka, Akihiro Yamashita, Atsushi Saito, Hideaki Sawai, Jun Murotsuki, Hirofumi Ohashi, Toshio Okamoto, Gen Nishimura, Kazunori Imaizumi, Noriyuki Tsumaki, (2015) Modeling type II collagenopathy skeletal dysplasia by directed conversion and induced pluripotent stem cells. Human Molecular Genetics 24 (2):299–313

Mitsuru Mizuno, Hisako Katano, Koji Otabe, Keiichiro Komori, Yukie Matsumoto, Shizuka Fujii, Nobutake Ozeki, Kunikazu Tsuji, Hideyuki Koga, Takeshi Muneta, Akifumi Matsuyama, Ichiro Sekiya, (2015) Platelet-derived growth factor (PDGF)-AA/AB in human serum are potential indicators of the proliferative capacity of human synovial mesenchymal stem cells. Stem Cell Research & Therapy 6 (1)

Mumme M, Steinitz A, Nuss KM, Klein K, Feliciano S, Kronen P, Jakob M, von Rechenberg B, Martin I, Barbero A, Pelttari K (2016a) Regenerative potential of tissue-engineered nasal chondrocytes in goat articular cartilage defects. Tissue Eng Part A 22:1286–1295

Mumme M, Barbero A, Miot S, Wixmerten A, Feliciano S, Wolf F, Asnaghi MA, Baumhoer D, Bieri O, Kretzschmar M, Pagenstert G, Haug M, Schaefer DJ, Martin I, Jakob M (2016b) Nasal chondrocytes-based engineered autologous cartilage tissue for the repair of articular cartilage defects: an observational first-in-human trial. Lancet 388:1985–1994

Mundy C, Yasuda T, Kinumatsu T, Yamaguchi Y, Iwamoto M, Enomoto-Iwamoto M, Koyama E, Pacifici M (2011) Synovial joint formation requires local Ext1 expression and heparan sulfate production in developing mouse embryo limbs and spine. Dev Biol 351:70–81

Muraglia A, Todeschi MR, Papait A, Poggi A, Spanò R, Strada P, Cancedda R, Mastrogiacomo M (2015) Combined platelet and plasma derivatives enhance proliferation of stem/progenitor cells maintaining their differentiation potential. Cytotherapy 17:1793–1806

Muraglia A, Cancedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113(Pt 7):1161–1166

Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48:3464–3474

Naumann A, Rotter N, Bujia J, Aigner J (1998) Tissue engineering of autologous cartilage transplants for rhinology. Am J Rhinol 12:59–63

Nagai T, Sato M, Kutsuna T, Kokubo M, Ebihara G, Ohta N, Mochida J (2010) Intravenous administration of anti-vascular endothelial growth factor humanized monoclonal antibody bevacizumab improves articular cartilage repair. Arthritis Res Ther 12:R178

Nakata K, Shino K, Hamada M, Mae T, Miyama T, Shinjo H, Horibe S, Tada K, Ochi T, Yoshikawa H (2001) Human meniscus cell: characterization of the primary culture and use for tissue engineering. Clin Orthop Relat Res 391:S208–S218

Nam HY, Karunanithi P, Loo WC, Naveen S, Chen H, Hussin P, Chan L, Kamarul T (2013) The effects of staged intra-articular injection of cultured autologous mesenchymal stromal cells on the repair of damaged cartilage: a pilot study in caprine model. Arthritis Res Ther 15:R129

Narcisi R, Cleary MA, Brama PAJ, Hoogduijn MJ, Tüysüz N, Berge ten D, van Osch GJVM (2015) Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. Stem Cell Reports 4:459–472

Nazempour A, Quisenberry CR, Van Wie BJ, Abu-Lail NI (2016) Nanomechanics of engineered articular cartilage: synergistic influences of transforming growth factor-beta 3 and oscillating pressure. J Nanosci Nanotechnol 16:3136–3145

Nazor KL, Altun G, Lynch C, Tran H, Harness JV, Slavin I, Garitaonandia I, Muller FJ, Wang YC, Boscolo FS, Fakunle E, Dumevska B, Lee S, Park HS, Olee T, D'Lima DD, Semechkin R, Parast MM, Galat V, Laslett AL, Schmidt U, Keirstead HS, Loring JF, Laurent LC (2012) Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. Cell Stem Cell 10(5):620–634

Nevo Z, Horwitz AL, Dorfmann A (1972) Synthesis of chondromucoprotein by chondrocytes in suspension culture. Dev Biol 28:219–228

Ning Ma, Baojian Liao, Hui Zhang, Linli Wang, Yongli Shan, Yanting Xue, Ke Huang, Shubin Chen, Xiaoxiao Zhou, Yang Chen, Duanqing Pei, Guangjin Pan, (2013) Transcription Activator-like Effector Nuclease (TALEN)-mediated Gene Correction in Integration-free Î²-Thalassemia Induced Pluripotent Stem Cells. Journal of Biological Chemistry 288 (48):34671–34679

Nishida Y, Knudson CB, Eger W, Kuettner KE, Knudson W (2000) Osteogenic protein 1 stimulates cell-associated matrix assembly by normal human articular chondrocytes - up-regulation of hyaluronan synthase,CD44, and aggrecan. Arthritis Rheum 43:206–214

Ni Y, Jiang Y, Wen J, Shao Z, Chen X, Sun S, Yu H, Li W (2014) Construction of a functional silk-based biomaterial complex with immortalized chondrocytes in vivo. J Biomed Mater Res A 102:1071–1078

Niemeyer P, Porichis S, Steinwachs M, Erggelet C, Kreuz PC, Schmal H, Uhl M, Ghanem N, Südkamp NP, Salzmann G (2014) Long-term outcomes after first-generation autologous chondrocyte implantation for cartilage defects of the knee. Am J Sports Med 42:150–157

Nieminen R, Korhonen R, Moilanen T, Clark AR, Moilanen E (2010) Aurothiomalate inhibits cyclooxygenase 2, matrix metalloproteinase 3, and interleukin-6 expression in chondrocytes by increasing MAPK phosphatase 1 expression and decreasing p38 phosphorylation: MAPK phosphatase 1 as a novel target for antirheumatic drugs. Arthritis Rheum 62:1650–1659

Norby DP, Malemud CJ, Sokoloff L (1977) Differences in the collagen types synthesized by lapine articular chondrocytes in spinner and monolayer culture. Arthritis Rheum 20:709–716

Nowlan NC, Sharpe J, Roddy KA, Prendergast PJ, Murphy P (2010) Mechanobiology of embryonic skeletal development: insights from animal models. Birth Defect Res C 90:203–213

Nuernberger S, Cyran N, Albrecht C, Redl H, Vécsei V, Marlovits S (2011) The influence of scaffold architecture on chondrocyte distribution and behavior in matrix-associated chondrocyte transplantation grafts. Biomaterials 32(4):1032–1040

Olivotto E, Borzi RM, Vitellozzi R, Pagani S, Facchini A, Battistelli M, Penzo M, Li X, Flamigni F, Li J, Falcieri E, Facchini A, Marcu KB (2008) Differential requirements for IKKalpha and IKKbeta in the differentiation of primary human osteoarthritic chondrocytes. Arthritis Rheum 58(1):227–239

Olivotto E, Otero M, Astolfi A, Platano D, Facchini A, Pagani S, Flamigni F, Facchini A, Goldring MB, Borzi RM, Marcu KB (2013) IKKalpha/CHUK regulates extracellular matrix remodeling independent of its kinase activity to facilitate articular chondrocyte differentiation. PLoS One 8 (9):e73024

Olsen BR, Reginato AM, Wang W (2000) Bone Development. Annu Rev Cell Dev Biol 16:191–220

Orozco L, Munar A, Soler R, Alberca M, Soler F, Huguet M, Sentís J, Sánchez A, García-Sancho J (2013) Treatment of knee osteoarthritis with autologous mesenchymal stem cells. Transplantation 95:1535–1541

Osaki M, Tan L, Choy BK, Yoshida Y, Cheah KS, Auron PE, Goldring MB (2003) The TATA-containing core promoter of the type II collagen gene (COL2A1) is the target of interferon-gamma-mediated inhibition in human chondrocytes: requirement for Stat1 alpha, Jak1 and Jak2. Biochem J 369(Pt 1):103–115

Otero M, Favero M, Dragomir C, Hachem KE, Hashimoto K, Plumb DA, Goldring MB (2012a) Human chondrocyte cultures as models of cartilage-specific gene regulation. Methods Mol Biol 806:301–336

Otero M, Plumb DA, Tsuchimochi K, Dragomir CL, Hashimoto K, Peng H, Olivotto E, Bevilacqua M, Tan L, Yang Z, Zhan Y, Oettgen P, Li Y, Marcu KB, Goldring MB (2012b) E74-like factor 3 (ELF3) impacts on matrix metalloproteinase 13 (MMP13) transcriptional control in articular chondrocytes under proinflammatory stress. J Biol Chem 287:3559–3572

Oxford JT, Doege KJ, Horton WE Jr, Morris NP (1994) Characterization of type II and type XI collagen synthesis by an immortalized rat chondrocyte cell line (IRC) having a low level of type II collagen mRNA expression. Exp Cell Res 213:28–36

Oyajobi BO, Frazer A, Hollander AP, Graveley RM, Xu C, Houghton A, Hatton PV, Russell RG, Stringer BM (1998) Expression of type X collagen and matrix calcification in three-dimensional cultures of immortalized temperature-sensitive chondrocytes derived from adult human articular cartilage. J Bone Miner Res 13:432–442

Pacifici M, Koyama E, Iwamoto M (2005) Mechanisms of synovial joint and articular cartilage formation: recent advances, but many lingering mysteries. Birth Defect Res C 75:237–248

Pacifici M, Koyama E, Shibukawa Y, Wu C, Tamamura Y, Enomoto-Iwamoto M, Iwamoto M (2006) Cellular and molecular mechanisms of synovial joint and articular cartilage formation. Ann N Y Acad Sci 1068:74–86

Panadero JA, Lanceros-Mendez S, Ribelles JLG (2016) Differentiation of mesenchymal stem cells for cartilage tissue engineering: individual and synergetic effects of three-dimensional environment and mechanical loading. Acta Biomater 33:1–12

Papadimitropoulos A, Piccinini E, Brachat S, Braccini A, Wendt D, Barbero A, Jacobi C, Martin I (2014) Expansion of human mesenchymal stromal cells from fresh bone marrow in a 3D scaffold-based system under direct perfusion. PLoS One 9:e102359

Park SS, Jin HR, Chi DH, Taylor RS (2004) Characteristics of tissue-engineered cartilage from human auricular chondrocytes. Biomaterials 25:2363–2369

Passaretti D, Silverman RP, Huang W, Kirchhoff CH, Ashiku S, Randolph MA, Yaremchuk MJ (2001) Cultured chondrocytes produce injectable tissue-engineered cartilage in hydrogel polymer. Tissue Eng 7:805–815

Patrick Cahan, George Q. Daley, (2013) Origins and implications of pluripotent stem cell variability and heterogeneity. Nature Reviews Molecular Cell Biology 14 (6):357–368

Pazin DE, Gamer LW, Cox KA, Rosen V (2012) Molecular profiling of synovial joints: use of microarray analysis to identify factors that direct the development of the knee and elbow. Dev Dyn 241:1816–1826

Pei M, Solchaga LA, Seidel J, Zeng L, Vunjak-Novakovic G, Caplan AI, Freed LE (2002) Bioreactors mediate the effectiveness of tissue engineering scaffolds. FASEB J 16:1691–1694

Pelttari K, Pippenger B, Mumme M, Feliciano S, Scotti C, Mainil-Varlet P, Procino A, RB v, Schwamborn T, Jakob M, Cillo C, Barbero A, Martin I (2014) Adult human neural crest-derived cells for articular cartilage repair. Sci Transl Med 6:251ra119

Peng H, Tan L, Osaki M, Zhan Y, Ijiri K, Tsuchimochi K, Otero M, Wang H, Choy BK, Grall FT, Gu X, Libermann TA, Oettgen P, Goldring MB (2008) ESE-1 is a potent repressor of type II collagen gene (COL2A1) transcription in human chondrocytes. J Cell Physiol 215(2):562–573

Penuela L, Wolf F, Raiteri R, Wendt D, Martin I, Barbero A (2014) Atomic force microscopy to investigate spatial patterns of response to interleukin-1beta in engineered cartilage tissue elasticity. J Biomech 47:2157–2164

Peterson L, Vasiliadis HS, Brittberg M, Lindahl A (2010) Autologous chondrocyte implantation: a long-term follow-up. Am J Sports Med 38:1117–1124

Piera-Velazquez S, Jimenez SA, Stokes D (2002) Increased life span of human osteoarthritic chondrocytes by exogenous expression of telomerase. Arthritis Rheum 46(3):683–693

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

Pleumeekers MM, Nimeskern L, Koevoet WL, Kops N, Poublon RM, Stok KS, van Osch GJ (2014) The in vitro and in vivo capacity of culture-expanded human cells from several sources encapsulated in alginate to form cartilage. Eur Cell Mater 27:264–280. discussion 278–80:264–280

Pomerantseva I, Bichara DA, Tseng A, Cronce MJ, Cervantes TM, Kimura AM, Neville CM, Roscioli N, Vacanti JP, Randolph MA, Sundback CA (2016) Ear-shaped stable auricular cartilage engineered from extensively expanded chondrocytes in an immunocompetent experimental animal model. Tissue Eng Part A 22:197–207

Popko M, Bleys RL, De Groot JW, Huizing EH (2007) Histological structure of the nasal cartilages and their perichondrial envelope. I. The septal and lobular cartilage. Rhinology 45:148–152

Quinn TM, Hunziker EB, Häuselmann HJ (2005) Variation of cell and matrix morphologies in articular cartilage among locations in the adult human knee. Osteoarthr Cartil 13:672–678

Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 30:783–791

Rapko S, Parker A, Mortelliti C, Duguay S, Wolfe L (2010) OR15: novel identity and potency quality control assays for autologous chondrocyte implantation. Orthop Proc 92-B(Suppl):204

Ray A, Singh PN, Sohaskey ML, Harland RM, Bandyopadhyay A (2015) Precise spatial restriction of BMP signaling is essential for articular cartilage differentiation. Development 142:1169–1179

Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB (2000) Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. Arthritis Rheum 43:2189–2201

Rodriguez A, Cao YL, Ibarra C, Pap S, Vacanti M, Eavey RD, Vacanti CA (1999) Characteristics of cartilage engineered from human pediatric auricular cartilage. Plast Reconstr Surg 103:1111–1119

Roger Croutze, Nadr Jomha, Hasan Uludag, Adetola Adesida, (2013) Matrix forming characteristics of inner and outer human meniscus cells on 3D collagen scaffolds under normal and low oxygen tensions. BMC Musculoskeletal Disorders 14 (1)

Rotter N, Bonassar LJ, Tobias G, Lebl M, Roy AK, Vacanti CA (2002) Age dependence of biochemical and biomechanical properties of tissue-engineered human septal cartilage. Biomaterials 23:3087–3094

Roy R, Kohles SS, Zaporojan V, Peretti GM, Randolph MA, Xu J, Bonassar LJ (2004) Analysis of bending behavior of native and engineered auricular and costal cartilage. J Biomed Mater Res A 68:597–602

Saadeh PB, Mehrara BJ, Steinbrech DS, Dudziak ME, Greenwald JA, Luchs JS, Spector JA, Ueno H, Gittes GK, Longaker MT (1999) Human cartilage engineering: chondrocyte extraction, proliferation, and characterization for construct development. Ann Plast Surg 42:509–513

Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131:324–336

Sah RL, Chen AC, Grodzinsky AJ, Trippel SB (1994) Differential-effects of bfgf and igf-i on matrix metabolism in calf and adult bovine cartilage explants. Arch Biochem Biophys 308:137–147

Saim AB, Cao Y, Weng Y, Chang CN, Vacanti MA, Vacanti CA, Eavey RD (2000) Engineering autogenous cartilage in the shape of a helix using an injectable hydrogel scaffold. Laryngoscope 110:1694–1697

Saliken DJ, Mulet-Sierra A, Jomha NM, Adesida AB (2012) Decreased hypertrophic differentiation accompanies enhanced matrix formation in co-cultures of outer meniscus cells with bone marrow mesenchymal stromal cells. Arthritis Res Ther 14:R153

Saris D, Price A, Widuchowski W, Bertrand-Marchand M, Caron J, Drogset JO, Emans P, Podskubka A, Tsuchida A, Kili S, Levine D, Brittberg M (2014) SUMMIT study group. Matrix-applied characterized autologous cultured chondrocytes versus microfracture: two year follow-up of a prospective randomized trial. Am J Sports Med 42:1384–1394

Sato M, Uchida K, Nakajima H, Miyazaki T, Guerrero AR, Watanabe S, Roberts S, Baba H (2012) Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. Arthritis Res Ther 14:R3

Saw K-Y, Hussin P, Loke S-C, Azam M, Chen H-C, Tay Y-G, Low S, Wallin K-L, Ragavanaidu K (2009) Articular cartilage regeneration with autologous marrow aspirate and hyaluronic acid: an experimental study in a goat model. Arthroscopy 25:1391–1400

Sawada R, Ito T, Tsuchiya T (2006) Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells. J Artif Organs 9:179–184

Schachar NS, Cucheran DJ, McGann LE, Novak KA, Frank CB (1994) Metabolic-activity of bovine articular-cartilage during refrigerated storage. J Orthop Res 12:15–20

Schneider U, Andereya S (2003) First results of a prospective randomized clinical trial on traditional chondrocyte transplantation vs CaReS-technology. Z Orthop Ihre Grenzgeb 141:496–497

Schultz-Coulon HJ, Eckermeier L (1976) Postnatal growth of nasal septum. Acta Otolaryngol 24:283–286

Scott MA, Levi B, Askarinam A, Nguyen A, Rackohn T, Ting K, Soo C, James AW (2012) Brief review of models of ectopic bone formation. Stem Cells Dev 21:655–667

Scotti C, Osmokrovic A, Wolf F, Miot S, Peretti GM, Barbero A, Martin I (2012) Response of human engineered cartilage based on articular or nasal chondrocytes to interleukin-1beta and low oxygen. Tissue Eng Part A 18:362–372

Sevin K, Askar I, Saray A, Yormuk E (2000) Exposure of high-density porous polyethylene (Medpor) used for contour restoration and treatment. Br J Oral Maxillofac Surg 38:44–49

Shafiee A, Kabiri M, Ahmadbeigi N, Yazdani SO, Mojtahed M, Amanpour S, Soleimani M (2011) Nasal septum-derived multipotent progenitors: a potent source for stem cell-based regenerative medicine. Stem Cells Dev 20:2077–2091

Shang J, Liu H, Li J, Zhou Y (2014) Roles of hypoxia during the chondrogenic differentiation of mesenchymal stem cells. Curr Stem Cell Res Ther 9:141–147

Shieh SJ, Terada S, Vacanti JP (2004) Tissue engineering auricular reconstruction: in vitro and in vivo studies. Biomaterials 25:1545–1557

Shimada H, Otero M, Tsuchimochi K, Yamasaki S, Sakakima H, Matsuda F, Sakasegawa M, Setoguchi T, Xu L, Goldring MB, Tanimoto A, Komiya S, Ijiri K (2016) CCAAT/enhancer binding protein beta (C/EBPbeta) regulates the transcription of growth arrest and DNA damage-inducible protein 45 beta (GADD45beta) in articular chondrocytes. Pathol Res Pract 212:302–309

Shwartz Y, Viukov S, Krief S, Zelzer E (2016) Joint development involves a continuous influx of Gdf5-positive cells. Cell Rep 15:2577–2587

Siegel NS, Gliklich RE, Taghizadeh F, Chang Y (2000) Outcomes of septoplasty. Otolaryngol Head Neck Surg 122:228–232

Singer NG, Caplan AI (2011) Mesenchymal stem cells: mechanisms of inflammation. Annu Rev Pathol 6:457–478

Solchaga LA, Penick K, Goldberg VM, Caplan AI, Welter JF (2010) Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. Tissue Eng Part A 16:1009–1019

Soler R, Orozco L, Munar A, Huguet M, López R, Vives J, Coll R, Codinach M, Garcia-Lopez J (2016) Final results of a phase I-II trial using ex vivo expanded autologous mesenchymal stromal cells for the treatment of osteoarthritis of the knee confirming safety and suggesting cartilage regeneration. Knee 23:647–654

Solvig Diederichs, Rocky S. Tuan, (2014) Functional Comparison of Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Cells and Bone Marrow-Derived Mesenchymal Stromal Cells from the Same Donor. Stem Cells and Development 23 (14):1594–1610

Somoza RA, Welter JF, Correa D, Caplan AI (2014) Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. Tissue Eng Part B Rev 20:596–608

Spalding T, Almqvist F, Brittberg M, Cole B, Farr J , Hosea T, Imhoff A, Mandelbaum B, Nehrer S, Richmond J (2011) The CAIS project; European multicentre randomised controlled pilot study of a one stage procedure for cell based cartilage repair. Orthopaedic Proceedings. 93-B no. SUPP III 292

Später D, Hill TP, Gruber M, Hartmann C (2006) Role of canonical Wnt-signalling in joint formation. Eur Cell Mater 12:71–80

Sridhar BV, Doyle NR, Randolph MA, Anseth KS (2014) Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production. J Biomed Mater Res A 102:4464–4472

Steimberg N, Viengchareun S, Biehlmann F, Guenal I, Mignotte B, Adolphe M, Thenet S (1999) SV40 large T antigen expression driven by col2a1 regulatory sequences immortalizes articular chondrocytes but does not allow stabilization of type II collagen expression. Exp Cell Res 249 (2):248–259

Steward AJ, Kelly DJ, Wagner DR (2016) Purinergic Signaling regulates the transforming growth factor-β3-induced Chondrogenic response of mesenchymal stem cells to hydrostatic pressure. Tissue Eng Part A 22:831–839

Storm EE, Kingsley DM (1996) Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. Development 122:3969–3979

Surridge AK, Rodgers UR, Swingler TE, Davidson RK, Kevorkian L, Norton R, Waters JG, Goldring MB, Parker AE, Clark IM (2009) Characterization and regulation of ADAMTS-16. Matrix Biol 28(7):416–424

Takazawa Y, Nifuji A, Mataga N, Yamauchi Y, Kurosawa H, Noda M (1999) Articular cartilage cells immortalized by a temperature sensitive mutant of SV40 large T antigen survive and form cartilage tissue in articular cartilage environment. J Cell Biochem 75(2):338–345

Takigawa M, Pan HO, Kinoshita A, Tajima K, Takano Y (1991) Establishment from a human chondrosarcoma of a new immortal cell line with high tumorigenicity in vivo, which is able to form proteoglycan-rich cartilage-like nodules and to respond to insulin in vitro. Int J Cancer 48:717–725

Tan L, Peng H, Osaki M, Choy BK, Auron PE, Sandell LJ, Goldring MB (2003) Egr-1 mediates transcriptional repression of COL2A1 promoter activity by interleukin-1beta. J Biol Chem 278 (20):17688–17700

Tan Y, Zhang Y, Pei M (2010) Meniscus reconstruction through coculturing meniscus cells with synovium-derived stem cells on small intestine submucosa – a pilot study to engineer meniscus tissue constructs. Tissue Eng Part A 16:67–79

Tavassoli M, Crosby WH (1968) Transplantation of marrow to extramedullary sites. Science 161:54–56

Tay AG, Farhadi J, Suetterlin R, Pierer G, Heberer M, Martin I (2004) Cell yield, proliferation, and postexpansion differentiation capacity of human ear, nasal, and rib chondrocytes. Tissue Eng 10:762–770

Tenneille E Ludwig, Veit Bergendahl, Mark E Levenstein, Junying Yu, Mitchell D Probasco, James A Thomson, (2006) Feeder-independent culture of human embryonic stem cells. Nature Methods 3 (8):637–646

Teo BJX, Buhary K, Tai B-C, Hui JH (2013) Cell-based therapy improves function in adolescents and young adults with patellar osteochondritis dissecans. Clin Orthop Relat Res 471:1152–1158

Terada S, Fuchs JR, Yoshimoto H, Fauza DO, Vacanti JP (2005) In vitro cartilage regeneration from proliferated adult elastic chondrocytes. Ann Plast Surg 55:196–201

Tew SR, Li Y, Pothacharoen P, Tweats LM, Hawkins RE, Hardingham TE (2005) Retroviral transduction with SOX9 enhances re-expression of the chondrocyte phenotype in passaged osteoarthritic human articular chondrocytes. Osteoarthr Cartil 13:80–89

Tew SR, Murdoch AD, Rauchenberg RP, Hardingham TE (2008) Cellular methods in cartilage research: primary human chondrocytes in culture and chondrogenesis in human bone marrow stem cells. Methods 45:2–9

Tew SR, Peffers MJ, McKay TR, Lowe ET, Khan WS, Hardingham TE, Clegg PD (2009) Hyperosmolarity regulates SOX9 mRNA posttranscriptionally in human articular chondrocytes. Am J Phys Cell Phys 297:C898–C906

Ting V, Sims CD, Brecht LE, McCarthy JG, Kasabian AK, Connelly PR, Elisseeff J, Gittes GK, Longaker MT (1998) In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes. Ann Plast Surg 40:413–420. discussion 20–21

Thenet S, Benya PD, Demignot S, Feunteun J, Adolphe M (1992) SV40-immortalization of rabbit articular chondrocytes: alteration of differentiated functions. J Cell Physiol 150:158–167

Thomas B, Thirion S, Humbert L, Tan L, Goldring MB, Bereziat G, Berenbaum F (2002) Differentiation regulates interleukin-1beta-induced cyclo-oxygenase-2 in human articular chondrocytes: role of p38 mitogen-activated protein kinase. Biochem J 362(Pt 2): 367–373

Thomson JA, Embryonic Stem Cell Lines Derived from Human Blastocysts. Science 282 (5391):1145–1147

Toegel S, Wu SQ, Piana C, Unger FM, Wirth M, Goldring MB, Gabor F, Viernstein H (2008) Comparison between chondroprotective effects of glucosamine, curcumin, and diacerein in IL-1beta-stimulated C-28/I2 chondrocytes. Osteoarthr Cartil 16:1205–1212

Toghraie F, Razmkhah M, Gholipour MA, Faghih Z, Chenari N, Torabi Nezhad S, Nazhvani Dehghani S, Ghaderi A (2012) Scaffold-free adipose-derived stem cells (ASCs) improve experimentally induced osteoarthritis in rabbits. Arch Iran Med 15:495–499

Toghraie FS, Chenari N, Gholipour MA, Faghih Z, Torabinejad S, Dehghani S, Ghaderi A (2011) Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in rabbit. Knee 18:71–75

Tormin A, Li O, Brune JC, Walsh S, Schütz B, Ehinger M, Ditzel N, Kassem M, Scheding S (2011) CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. Blood 117:5067–5077

Tseng A, Pomerantseva I, Cronce MJ, Kimura AM, Neville CM, Randolph MA, Vacanti JP, Sundback CA (2014) Extensively expanded auricular chondrocytes form Neocartilage in vivo. Cartilage 5:241–251

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

Turner M, Leslie S, Martin NG, Peschanski M, Rao M, Taylor CJ, Trounson A, Turner D, Yamanaka S, Wilmut I (2013) Toward the development of a global induced pluripotent stem cell library. Cell Stem Cell 13(4):382–384

Ulrich-Vinther M, Maloney MD, Goater JJ, Soballe K, Goldring MB, O'Keefe RJ, Schwarz EM (2002) Light-activated gene transduction enhances adeno-associated virus vector-mediated gene expression in human articular chondrocytes. Arthritis Rheum 46(8):2095–2104

Upton ML, Chen J, Setton LA (2006) Region-specific constitutive gene expression in the adult porcine meniscus. J Orthop Res 24:1562–1570

Vanlauwe J, Saris DB, Victor J, Almqvist KF, Bellemans J, Luyten FP, TIG/ACT/01/2000&EXT Study Group (2011) Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters. Am J Sports Med 39:2566–2574

Van Osch GJ, Marijnissen WJ, van der Veen SW, Verwoerd-Verhoef HL (2001a) The potency of culture-expanded nasal septum chondrocytes for tissue engineering of cartilage. Am J Rhinol 15:187–192

Van Osch GJ, van der Veen SW, Verwoerd-Verhoef HL (2001b) In vitro redifferentiation of culture expanded rabbit and human auricular chondrocytes for cartilage reconstruction. Plast Reconstr Surg 107:433–440

Van Osch GJ, Mandl EW, Jahr H, Koevoet W, Nolst-Trenité G, Verhaar JA (2004) Considerations on the use of ear chondrocytes as donor chondrocytes for cartilage tissue engineering. Biorheology 41:411–421

Verdonk PCM, Forsyth RG, Wang J, Almqvist KF, Verdonk R, Veys EM, Verbruggen G (2005) Characterisation of human knee meniscus cell phenotype. Osteoarthr Cartil 13(7):548–560

Vinatier C, Magne D, Moreau A, Gauthier O, Malard O, Vignes-Colombeix C, Daculsi G, Weiss P, Guicheux J (2007) Engineering cartilage with human nasal chondrocytes and a silanized hydroxypropyl methylcellulose hydrogel. J Biomed Mater Res A 80:66–74

Visna P, Pasa L, Cizmár I, Hart R, Hoch J (2004) Treatment of deep cartilage defects of the knee using autologous chondrograft transplantation and by abrasive techniques–a randomized controlled study. Acta Chir Belg 104:709–714

Wakitani S, Nawata M, Tensho K, Okabe T, Machida H, Ohgushi H (2007) Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees. J Tissue Eng Regen Med 1:74–79

Wei Niu, Weimin Guo, Shufeng Han, Yun Zhu, Shuyun Liu, Quanyi Guo, (2016) Cell-Based Strategies for Meniscus Tissue Engineering. Stem Cells International 2016:1–10

Weiss S, Hennig T, Bock R, Steck E, Richter W (2010) Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. J Cell Physiol 223:84–93

Williams R, Khan IM, Richardson K, Nelson L, McCarthy HE, Analbelsi T, Singhrao SK, Dowthwaite GP, Jones RE, Baird DM, Lewis H, Roberts S, Shaw HM, Dudhia J, Fairclough J, Briggs T, Archer CW (2010) Identification and clonal characterisation of a progenitor cell subpopulation in normal human articular cartilage. PLoS One 5:e13246

Wolf F, Haug M, Farhadi J, Candrian C, Martin I, Barbero A (2008) A low percentage of autologous serum can replace bovine serum to engineer human nasal cartilage. Eur Cell Mater 15:1–10.:1-10

Wu L, Leijten JC, Georgi N, Post JN, van Blitterswijk CA, Karperien M (2011) Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation. Tissue Eng Part A 17:1425–1436

Xing Zhao, Nathaniel S. Hwang, David A. Bichara, Daniel B. Saris, Jos Malda, Joseph P. Vacanti, Irina Pomerantseva, Cathryn A. Sundback, Robert Langer, Daniel G. Anderson, Mark A. Randolph, (2017) Chondrogenesis by bone marrow-derived mesenchymal stem cells grown in chondrocyte-conditioned medium for auricular reconstruction. Journal of Tissue Engineering and Regenerative Medicine 11 (10):2763–2773

Xu JW, Nazzal J, Peretti GM, Kirchhoff CH, Randolph MA, Yaremchuk MJ (2001) Tissue engineered cartilage composite with expanded polytetrafluoroethylene membrane. Ann Plast Surg 46:527–532

Xu JW, Zaporojan V, Peretti GM, Roses RE, Morse KB, Roy AK, Mesa JM, Randolph MA, Bonassar LJ, Yaremchuk MJ (2004) Injectable tissue-engineered cartilage with different chondrocyte sources. Plast Reconstr Surg 113:1361–1371

Xu Y (2015) Tissue engineering of human nasal alar cartilage precisely by using three-dimensional printing

Xu L, Peng H, Wu D, Hu K, Goldring MB, Olsen BR, Li Y (2005) Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. J Biol Chem 280(1):548–555

Yanaga H, Imai K, Fujimoto T, Yanaga K (2009) Generating ears from cultured autologous auricular chondrocytes by using two-stage implantation in treatment of microtia. Plast Reconstr Surg 124:817–825

Yilin Cao, Joseph P. Vacanti, Keith T. Paige, Joseph Upton, Charles A. Vacanti, Transplantation of Chondrocytes Utilizing a Polymer-Cell Construct to Produce Tissue-Engineered Cartilage in the Shape of a Human Ear. Plastic and Reconstructive Surgery 100 (2):297–302

Zamperone A, Pietronave S, Merlin S, Colangelo D, Ranaldo G, Medico E, Di Scipio F, Berta GN, Follenzi A, Prat M (2013) Isolation and characterization of a spontaneously immortalized multipotent mesenchymal cell line derived from mouse subcutaneous adipose tissue. Stem Cells Dev 22:2873–2884

Zeifang F, Oberle D, Nierhoff C, Richter W, Moradi B, Schmitt H (2010) Autologous chondrocyte implantation using the original periosteum-cover technique versus matrix-associated autologous chondrocyte implantation: a randomized clinical trial. Am J Sports Med 38:924–933

Zhang Q, Cigan AD, Marrero L, Lopreore C, Liu S, Ge D, Savoie FH, You Z (2011) Expression of doublecortin reveals articular chondrocyte lineage in mouse embryonic limbs. Genesis 49:75–82

Zhou L, Pomerantseva I, Bassett EK, Bowley CM, Zhao X, Bichara DA, Kulig KM, Vacanti JP, Randolph MA, Sundback CA (2011) Engineering ear constructs with a composite scaffold to maintain dimensions. Tissue Eng Part A 17:1573–1581

Zhu F, Wang P, Lee NH, Goldring MB, Konstantopoulos K (2010) Prolonged application of high fluid shear to chondrocytes recapitulates gene expression profiles associated with osteoarthritis. PLoS One 5(12):e15174

Zscharnack M, Hepp P, Richter R, Aigner T, Schulz R, Somerson J, Josten C, Bader A, Marquass B (2010) Repair of chronic osteochondral defects using predifferentiated mesenchymal stem cells in an ovine model. Am J Sports Med 38:1857–1869

Zwicky R, Muntener K, Goldring MB, Baici A (2002) Cathepsin B expression and down-regulation by gene silencing and antisense DNA in human chondrocytes. Biochem J 367:209–217

Periosteum Derived Cells in Skeletal Tissue Regeneration

Johanna Bolander, Tim Herpelinck, and Frank P. Luyten

Contents

J. Bolander · T. Herpelinck · F. P. Luyten (✉)
Tissue Engineering Laboratory, Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium

Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium
e-mail: johanna.bolander@kuleuven.be; tim.herpelinck@kuleuven.be; frank.luyten@uzleuven.be

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_6

Abstract

The field of skeletal tissue engineering has in recent years been transformed by the identification of specific skeletal progenitor cell populations and their role in bone fracture healing. Specifically, progenitor cells residing within the periosteum have been shown to be crucial for bone regeneration. Importantly, this is not a phenomenon performed by one common progenitor cell, instead, distinct skeletal progenitor cell populations have been identified within the periosteum, shown to also contribute to different aspects of tissue repair. These findings represent major steps in the field of regenerative medicine, since there is currently no reliable treatment for patients with a failing endogenous repair system. Therefore, insights regarding the specific cell populations and factors that steer their homing and differentiation *in vivo* can aid in the development of optimized and personalized engineered treatment strategies. In this chapter, we highlight the crucial role of periosteum-derived cells in bone development, homeostasis, and repair. We next provide an overview of the periosteum-residing skeletal progenitor cells identified so far and their role in bone regeneration. Subsequently, we discuss the required steps to isolate and expand periosteal cells *in vitro*, and the current state of the art in the use of periosteum-derived cells for bone formation and regeneration following the intramembranous, endochondral, or osteochondral tissue repair route. Finally, we present an overview of periosteal cells in the preclinical and clinical setting and discuss their future potential.

1 Introduction

Cell-based strategies in regenerative medicine have emerged as promising treatment alternatives when the patient's intrinsic regenerative potential is hampered (Bianco et al. 2013). Yet, the majority of developed products still faces difficulties in the translation of *in vitro* findings to more clinically relevant *in vivo* settings. It has been suggested that one of the major reasons for this is the limited attention paid to the mechanism of action in the natural healing process of the damaged tissue of interest (Bianco et al. 2013; Heathman et al. 2015). In general, the cell population(s) of choice provides the driving force of the "living" implants regenerative capacity. Thus, the success of a cell-based construct highly relies on the choice of (a) potent and reliable progenitor cell population(s) able to form the required tissue, together with the subsequent stimulatory cues to frame and support these cells. As a result, the current lack of convincing clinical success in available therapies may be an issue related to the lack of a solid scientific basis supporting a quality by design. To deal with this, the developmental engineering principle has been proposed and gained attention by fusing concepts from developmental biology with engineering principles (Ingber et al. 2006; Lenas et al. 2009a, b). The concept of developmental engineering initiated a paradigm shift in the field of regenerative medicine. Instead of developing constructs resembling the original healthy tissue, scientists have now started developing treatments recapitulating

events in healthy tissue regeneration in order to overcome the existing limitations present in a compromised environment.

A crucial aspect when designing a cell-based regenerative construct is defined as having sufficient knowledge with regards to the anatomical and physiological properties of the original tissue to be restored or replaced. Thereafter, critical factors required for the regeneration should be combined to develop a functional, engineered solution for a well-defined clinical problem. With this in mind, the chosen factors (i.e., cells, stimulatory factors, 3D matrix) used in the development of a cell-based construct should therefore be inspired by the essential or even crucial factors required and sufficient for a natural healing process.

Bones represent unique organs in the human body due to their capability to regenerate upon damage without scar tissue formation. However, when the fracture environment is compromised, for instance, due to the large size, the patient has an impediment of the natural repair system (disease, genetic disposition, poor life style) or the surrounding environment including vascularity is severely affected, additional repair strategies are required for successful healing. Examples include massive trauma, but can also arise secondary, such as after osteosarcoma resection surgery or in patients with co-morbidities such as diabetes, and may lead to the development of a nonunion. Unsuccessful treatment of a nonunion leads to significant loss of mobility and independence with substantial loss in quality of life. In addition, prolonged hospitalization and work incapacity primes towards a large economic burden for the patient as well as society. Unfortunately, prolonged failure to regenerate the defect may even lead to amputation of the limb.

In recent years it has been established that the periosteum is not only largely involved in bone strength maintenance, and its preservation is crucial for normal bone repair (Duchamp de Lageneste et al. 2018). Consequently, periosteal cells have gained an increasing interest from an engineering perspective for skeletal tissue engineering purposes. The inner cambium layer of periosteum contains a heterogeneous cell population including fibroblasts, endothelial, and progenitor cells. The population of progenitor cells can be further separated based on their fate commitment. Even though it can be hypothesized that the detailed identification of the specific populations of skeletal progenitor cells present in the periosteum has just started, several specific progenitor cell populations have recently been identified that contribute to intramembranous bone formation and endochondral fracture healing and are listed in Table 1. Upon isolation of periosteum derived cells (PDCs), the heterogeneous and plastic-adherent populations are expanded and exhibit great clonogenicity, growth, and differentiation capacity. Even though their bone formation potential has been confirmed to exceed those of the more commonly used progenitor cells such as bone marrow stromal cells or adipose tissue derived progenitor cells (Agata et al. 2007; Colnot 2009; Hayashi et al. 2008; Yoshimura et al. 2007), only a fraction of research publications in the field of skeletal tissue regeneration report the use of PDCs. This is most likely due to limited access.

The periosteum is easily accessible once a fracture occurs, but the periosteum's critical role in fracture healing limits the maximum size of the biopsy, in order not to jeopardize the healing. Therefore, the use of human periosteum derived cells for

Table 1 Overview of skeletal stem cells identified so far

Skeletal stem cells identified in mouse		
Candidate markers	Location of discovery	Reference
$CD45^-$ $Ter119^-$ $Sca1^+$ $PDGFR\alpha^+$	Perivascular cells	(Morikawa et al. 2009)
CD146	Perivascular cells	(Sacchetti et al. 2007)
Nestin (overlapping with LepR)	Perivascular cells	(Isern et al. 2014; Kunisaki et al. 2013; Mendez-Ferrer et al. 2010; Ono et al. 2014)
Grem1	Growth plate and metaphysis	(Worthley et al. 2015)
$CD45^-$ $Ter119^-$ $Tie2^-$ $AlphaV^+$ thy^-6C3^- $CD105^-$ $CD200^+$	Growth plate	(Chan et al. 2015)
PTHrP	Growth plate	(Mizuhashi et al. 2018)
CD73	Growth plate	(Newton et al. 2019)
Gli1	Growth plate	(Shi et al. 2017)
$Thy1^-$ $6C3^-$ CD200+, CD105−	Periosteum	(Debnath et al. 2018)
Postn	Periosteum	(Duchamp de Lageneste et al. 2018)
HoxA11	Periosteum	(Pineault et al. 2019)
$Mx1^+$ α-SMA^+	Periosteum	(Ortinau et al. 2019)
Skeletal stem cells identified in man		
$CD146^-$ $PDPN^+$ $CD73^+$ $CD164^+$	Growth plate	(Chan et al. 2018)

research purposes is limited to settings where an orthopedic surgeon or traumatologist is committed and able to collect a limited sample. *Post-mortem* collection can be an alternative, but requires the ability to collect the sample within 6 hours and requires additional administrative procedures and ethical approval in most countries. Upon successful collection and *in vitro* expansion of periosteum derived cells, several interesting and innovative routes have been undertaken *in vitro* and *in vivo* to utilize and understand the unique ability of PDCs to improve or restore the regenerative potential of bone defects.

2 Primary Cells: Periosteal Cells

The bony skeleton has a remarkable regenerative potential, generated by pools of skeletal stem and progenitor cells which differentiate into bone and cartilage to consolidate fractures. An essential source of these cells is the periosteum, the tissue which envelops nearly every bone. The periosteum is involved in bone growth, remodeling, and repair. In this chapter, we summarize the current knowledge on bone regeneration, the role of the skeletal stem and progenitor cells in this process, and the periosteum as a source thereof and their application in tissue engineering.

3 Developmental Origin

Bone is formed through two different processes. One involves the direct formation of bone within a membrane. This is referred to as primary, direct, or intramembranous ossification, which forms the flat bones of the skull, parts of the mandible and the lateral ends of the clavicles. The remainder of the axial and appendicular skeleton including the long bones is formed by secondary or endochondral ossification, a process involving the formation of a temporary cartilage template, which is subsequently replaced by bone.

During long bone development, undifferentiated mesenchymal cells condense into the shape of the bone. The mesenchymal condensations then undergo differentiation and become embedded in a cartilaginous matrix. At this point in time, the outline of the bone is in place but the future joint areas, the joint interzones, are still filled with cells. As the ephiphyseal end of the bone acquires its shape, the cells in the interzones undergo apoptosis, thereby forming the joint cavity and completing the blueprint of the prospective bone and joint. The cartilage *anlage* of the bone then undergoes radial and longitudinal expansion by interstitial and appositional growth, respectively. The cartilage template itself is enveloped by a cellular condensation of perichondrial cells. The expansion of the condensate is driven by proliferating chondrocytes, which give rise to columnar structures. Towards the end of the columns, the chondrocytes gradually undergo hypertrophy. Hypertrophic chondrocytes then enter apoptosis to leave lacunae in the matrix or transdifferentiate into osteoblasts. These lacunae are subsequently invaded by blood vessels, which provide essential nutrients, oxygen, and mesenchymal cells that differentiate into osteoblasts. The osteoblasts remodel the cartilaginous matrix and synthesize the first bone matrix, thereby forming the primary center of ossification. The bone lengthens as the proliferating chondrocytes progressively undergo hypertrophy, thereby moving up the ossification front. The perichondrium adjacent to the primary ossification center now forms the periosteum of the new long bone (Moreira et al. 2000) (Fig. 1a).

The periosteum itself is a double-layered tissue which envelops nearly every bone in the body (Dwek 2010) (Fig. 1b). It is connected to the bone by the fibers of Sharpey. The inner layer is highly osteogenic, while the outer fibrous layer is collagenous and poor in elastin fibers. Throughout the surface of the fibrous layer runs an extensive vascular and neural network, which in part runs through the bone cortex. The vasculature of the outer layer is one of the major blood supplies of the bone. Upon close examination of the outer periosteal layer, it can be further dissected into two substrata: the aforementioned highly innervated and vascularized layer and a deeper more fibroelastic region. This layer is located directly adjacent to the inner periosteal layer, contains more elastic fibers than the superficial layer, and is not very vascularized. The periosteal tendons are attached to this stratum.

The inner layer of the periosteum is densely populated with fibroblasts, progenitor cells, and osteoblasts (Allen et al. 2004). These osteoblasts directly line the cortex of the bone. Referencing the cambium layer of the trees, which is responsible for appositional growth and creates the characteristic ring pattern, the inner layer of

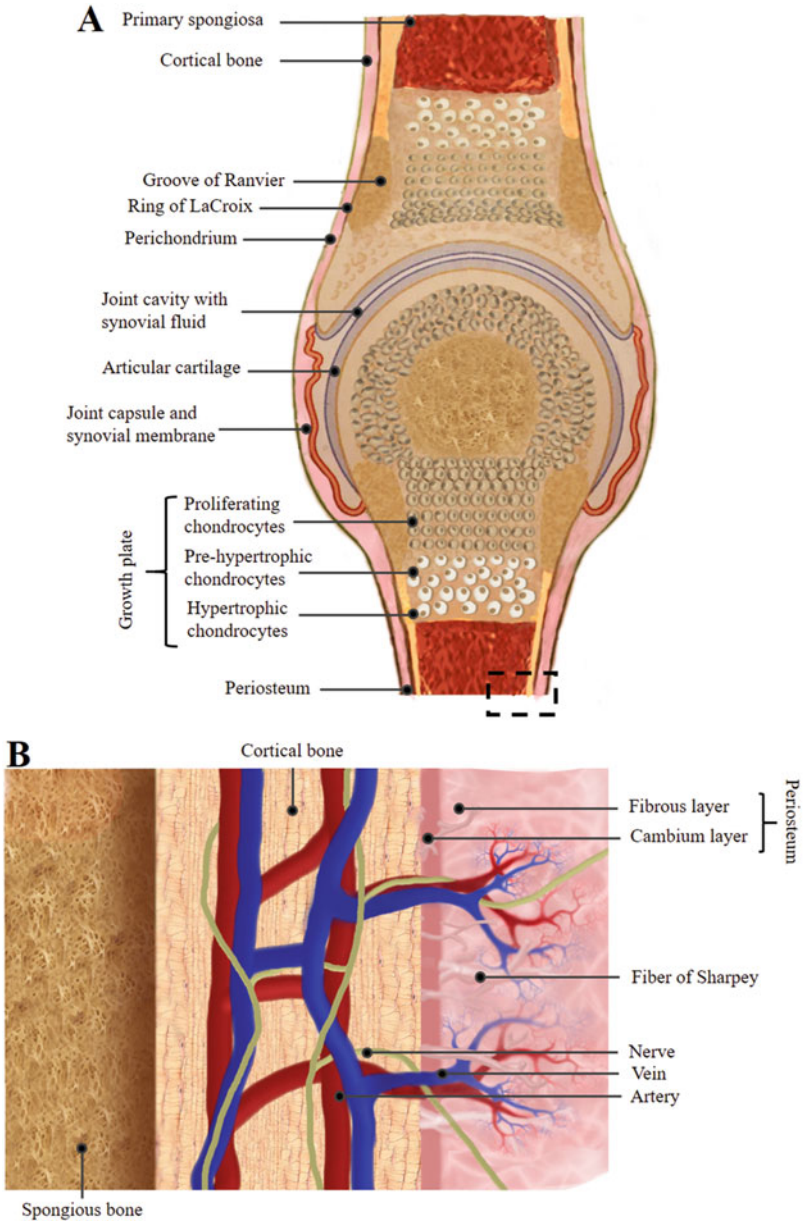


Fig. 1 Anatomy of joints and the periosteum. Schematic drawing of a synovial joint (**a**) An enlarged view of the boxed area is shown in (**b**) For clarity, the fibrous layer of the periosteum is folded backwards

the periosteum was named the cambial layer by Henri-Louis Duhamel in 1742. In a strikingly parallel fashion, the cambial layer of the periosteum is responsible for the radial growth of bones. The cambial layer also hosts a vascular and neural network. Resulting from the large presence of vessels, a significant number of pericytes can be found in this stratum of the periosteum (Diaz-Flores et al. 1992).

While the growth plate chondrocytes continue to lengthen the bone, the periosteal osteoblasts lining the cortex begin to deposit bony matrix to expand the radius of the bone by intramembranous ossification. Meanwhile, joint cavitation finishes at the interzones, leaving the articular surfaces uncovered. This allows the formation of articular cartilage (Archer et al. 2003). At the periphery of the cartilage anlage, the fibrous capsule of the joint is formed in a similar fashion as the perichondrium and periosteum condensed. Consequently, the fibrous joint capsule, the perichondrium, and the periosteum share similar developmental pathways in their formation.

The periosteum surrounds the diaphysis up to the groove of Ranvier, a circumferential groove in the periphery of the epiphyseal cartilage, where it provides both osteoblasts and chondrocytes for appositional growth. It thereby also encapsulates the perichondrium lining the epiphyseal growth plate, the ring of LaCroix, to provide additional structural integrity. Beyond the groove of Ranvier, the periosteum transitions into the perichondrium which is in turn continuous with the joint capsule (Davies 1963).

In later stages of development, blood vessels also invade the epiphyseal cartilage and a secondary ossification center is formed. As secondary centers of ossification are mostly intra-articular, they are not covered in periosteum, contrary to the primary centers of ossification.

Both the periosteum and the growth plates continue to increase the size of the bones until skeletal maturity is reached. At this point the growth plates undergo ossification, otherwise referred to as closure of the growth plates. The periosteum becomes relatively quiescent and takes on the role of a reservoir of osteoblasts and osteochondroprogenitors. Throughout life, these cells delicately interplay with bone resorbing osteoclasts to continuously remodel the bone cortex in response to mechanical loading.

4 Fracture Healing

The relative quiescence of the skeletal progenitor cells within the periosteum is abruptly terminated upon fracture of the bone. The degree of periosteal cell activation depends on the location of the fracture and its mechanical stability. If the fracture occurs in the metaphysis of the bone, the relative contribution of the periosteum to the healing process is rather limited, as the spongious bone of the metaphysis is rich in osteogenic bone marrow stromal cells. Diaphyseal fractures however are, if not

properly fixated, bridged by a healing response concerted primarily by the periosteum. Conversely, rigid fixation hampers the periosteal response (McKibbin 1978). Bone fracture healing involves most often both intramembranous and endochondral ossification and is generally described as a five-stage process.

4.1 Stage 1: Hematoma Formation

The hematoma is the crucial initial signaling center which initiates the cellular and molecular cascades of fracture healing, and its removal leads to defective bone healing (Mizuno et al. 1990). The hematoma is formed as a result of the rupture of blood vessels at the moment of trauma (Fig. 2a). At this point, the extrinsic coagulation cascade activates in response to the exposed subendothelial tissue factor (TF) (Broos et al. 2011). This leads to the activation of thrombin, which converts inactive fibrinogen into fibrin, which forms a cross-linked fibrin clot. Concomitantly, platelets activate and translocate their integrin receptors to the membrane. These have high affinity for fibrin, fibrinogen, von Willebrand Factor (vWF), thrombospondin, and fibronectin, leading to platelet aggregation. The platelets become trapped in the fibrin clot and degranulate. Inside platelet granules is a large store of mediators of inflammation such as cytokines, chemokines, and growth factors. Therefore, the stage of hematoma formation is accompanied by a phase of inflammation.

4.1.1 The Clearing of Debris

The release of the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα) recruits neutrophils, lymphocytes,

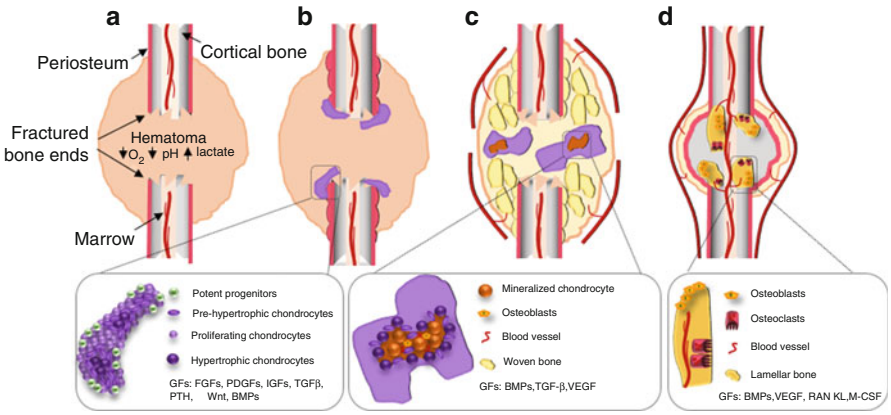


Fig. 2 The fracture healing cascade. Immediately after fracture a hematoma is formed which initiates fracture healing (**a**). Stem and progenitor cells in the periosteum undergo rapid expansion and begin to form a soft cartilaginous callus (**b**). The vascularized callus becomes weight-bearing as the hard callus is formed. (**c**) Cartilage is then removed and replaced by bone, later followed by the coupled remodeling of the newly formed bone (**d**)

and monocytes to the fracture site by promoting their extravasation. Neutrophils are the first to arrive to the defect site (Xian et al. 2004) and start clearing debris and dead cells by phagocytosis (Stuart and Hehir 1992). Additionally, neutrophils play an important role in preventing sepsis (Segal 2005).

At the same time, the damaged edges of the bone need to be eroded, meaning high numbers of osteoclasts are required. This is also mediated by the immune system, as receptor activator of nuclear factor kappa-B ligand (RANKL) produced by activated T-lymphocytes and NK cells can induce the differentiation of osteoclasts from monocytes and B-lymphocytes (Kong et al. 1999; Manabe et al. 2001).

4.1.2 Recruitment of Stem and Progenitor Cells

The inflammatory ligands released by the platelets and type 2 macrophages in the hematoma, specifically TNFα and stromal cell-derived factor 1 (SDF-1, CXCL12), recruit stromal and periosteal stem/progenitor cells to the site of tissue injury (Bocker et al. 2008; Kitaori et al. 2009). This recruitment is maintained by signals from the invading immune cells. The macrophage-derived chemokines MCP-1 and CXCL7, produced by NK cells, have both been found to be involved in recruiting progenitors from the periosteum, endosteum, and bone marrow (Almeida et al. 2016; Ishikawa et al. 2014) As a result, the initial migratory signals released by the degranulating activated platelets are sustained, albeit through different molecules, by the innate immune system. Concomitantly, interferon-gamma (IFN-γ) and TNFα released by NK cells and T-lymphocytes (Croitoru-Lamoury et al. 2011; Dorronsoro et al. 2014) activate the immunosuppressive functions of the stem and progenitor cells. The bestowal of immunosuppressive properties onto a cell type by cells of the immune system is referred to as licensing, which is used to control the inflammatory phase.

4.2 Stage 2: Reinnervation, Revascularization, and Formation of the Cartilaginous Callus

The growth factors initially released by platelets and later by macrophages in the hematoma induce activation and rapid proliferation of periosteal progenitors. In particular, the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) pathways initiate a rapid cell expansion in the cambial layer, resulting in thickening of the periosteum. Periosteal cells distal from the fracture differentiate into osteoblasts and drive intramembranous ossification to increase stability at the broken ends. Cambial cells proximal to the fracture invade the hematoma, where the fibrin clot acts as a temporary scaffold, and start producing cartilaginous matrix. The hematoma is then rapidly replaced by hyaline cartilage, formed primarily by PDCs. This cartilaginous structure represents the soft callus, a transient tissue which acts as a template for the further regeneration of the bone. It bridges the gap created by the fracture (Fig. 2b) and provides some stabilization of the fracture.

Concurrent with the formation of the callus, blood supply is restored through revascularization. This process is regulated by the angiopoietin and vascular endothelial growth factor (VEGF) pathways. The angiopoietins are vascular morphogenetic

proteins, which promote the ingrowth of new vessels from preexisting vessels in the periosteum (Lehmann et al. 2005). Neo-angiogenesis on the other hand is mainly regulated by the VEGF pathway and progresses differently depending on the fracture. In the absence of displacement, vessel sprouting mainly occurs from within the medullary arterial system. However, if major displacement occurs, the periosteum is the main source of new vessels, only followed by the medullary cavity later on (Rhinelander and Baragry 1962).

Reinnervation of the fracture site slightly precedes revascularization and is orchestrated by periosteal cells and macrophages. In response to nerve growth factor (NGF), periosteal nerve fibers begin to sprout dendrites and undergo duplication before invading the fracture callus (Li et al. 2019). Blood vessels and nerve fibers follow very similar paths in the skeleton (Bjurholm et al. 1988), but nerves are thought to provide the template of the branching pattern by attracting new vessels via VEGF signaling (Li et al. 2013).

4.3 Stage 3: Formation of the Hard Callus

Next, subperiosteal intramembranous ossification creates a bony hard callus, making the fracture callus mechanically stable and weight-bearing (Fig. 2c). At this time, the callus consists of two components: the hard and soft callus, where respectively intramembranous and endochondral ossification take place (Einhorn 1998; Schell et al. 2017).

4.4 Stage 4: Cartilage Removal and Bone Formation

Calcification of the soft callus follows a similar mechanism as endochondral ossification at the growth plate. Proliferating chondrocytes exit from mitosis, undergo hypertrophy, and produce pro-angiogenic ligands such as VEGF. The invading vessels bring with them perivascular cells that are osteoblast progenitors (Maes et al. 2010). This process leads to the transition of the callus into calcified cartilage, a structure which is nearly identical to the primary spongiosa found at the growth plate.

4.5 Stage 5: Coupled Remodeling

The callus is now fully composed of woven bone and surrounded by a new cortex. At this stage the bone enters the phase of coupled remodeling: osteoblasts deposit bone matrix to create a fully mechanically competent lamellar structure, while osteoclasts resorb excessive matrix from the transitional callus to restore the medullary cavity (Fig. 2d).

It is clear that endochondral fracture healing has strong parallels to the endochondral bone forming pathway during development. Both progress through a cartilage

intermediate, in which chondrocytes proliferate, swell, and die, to ultimately be replaced by bone. However, where in development the role of the periosteum is restricted to appositional growth by intramembranous ossification, its role becomes much more varied in endochondral fracture healing. It is the source of the osteochondral progenitors which form the cartilaginous soft callus and the osteogenic progenitors which largely form the hard callus. While the progenitor cells located in the bone marrow, BMSCs, actively contribute to the healing, their role is secondary to the PDCs. Because the PDCs are so crucial in fracture healing and are able to recapitulate bone formation as seen during development, they can be considered a prime cell source for skeletal tissue engineering and developmental engineering in particular (Lenas et al. 2009a, b).

5 Identification of Skeletal Stem Cells within the Periosteum

The ideal cell for a developmentally engineered implant for fracture repair is a skeletal stem cell (SSC), preferably of periosteal origin due to their critical role in fracture healing (Duchamp de Lageneste et al. 2018). By definition of a stem cell, this would mean a cell that is able to self-renew and differentiate into multiple cell fates, thereby contributing to tissue ontogeny, homeostasis and regeneration (Bianco and Robey 2015). In recent years, multiple postnatal, lineage-restricted SSCs have been identified in multiple compartments of the bone in both human and mouse.

Groundbreaking work in identifying the first bone marrow-derived cells capable of restoring cartilage, bone, stromal tissues, and the hematopoietic compartment was performed in the 1960s by Tavassoli and Crosby (Tavassoli and Crosby 1968). Twenty years later, the concept of multipotent cells, capable of *in vivo* self-renewal, was proposed (Owen and Friedenstein 1988), shortly thereafter dubbed mesenchymal stem cells (MSCs) (Caplan 1991). These cells were characterized as plastic-adherent, immunosuppressive, capable of *in vitro* colony formation, *in vitro* differentiation into the osteogenic, chondrogenic, and adipogenic lineage and *in vivo* self-renewal. They express the cell surface proteins CD73, CD90, and CD105, but no hematopoietic lineage markers.

Unfortunately, nonskeletal fibroblastic cells have also been labeled multipotent mesenchymal stem cells by their ability to make *in vitro* bone, cartilage, and fat (Via et al. 2012). The term MSC should be therefore be avoided for clarity issues. Moreover, it is fundamentally flawed, as no adult stem cell should be called a "mesenchymal" cell, since this term is reserved for an embryonic tissue. In current literature, MSCs have thus become a heterogeneous aggregate of plastic-adherent cells capable of tri-lineage differentiation *in vitro*, leading to great inconsistencies and the impediment of clinical applications based on stromal stem cell populations (Robey 2017). In light of the recent identification of *bona fide* SSCs within the skeleton, it has been proposed to completely abolish the term MSC and adopt the term bone marrow stromal cell (BMSC) to refer to the original "MSC" (Bianco and Robey 2015).

Skeletal stem cells have been described in the growth plate, in association with blood vessels as pericytes, the bone marrow, the periosteum, and fracture calluses. A multipotent perivascular population with *in vivo* self-renewal was described by Morikawa *et al.* as $CD45^{-}Ter119^{-}$ $Sca1^{+}$ $PDGFR\alpha^{+}$ cells, with *in vitro* osteogenic, chondrogenic, adipogenic clonogenicity (Morikawa et al. 2009). Other pericyte-like SSCs are found in the bone marrow and express the surface marker CD146 (Sacchetti et al. 2007). These cells are of subendothelial origin, are clonogenic, and give rise to a bone ossicle *in vivo* which supports hematopoiesis.

Multiple SSCs have been described in the murine bone marrow, although caution when interpreting these data is advised due to the absence of highly specific markers (Mendez-Ferrer et al. 2015). Nestin is commonly used to refer to bone marrow stromal stem cells. Indeed, in the bone marrow resides a self-renewing perivascular cell positive for Nestin, capable of osteochondral differentiation (Mendez-Ferrer et al. 2010). Later studies demonstrated a partial overlap between a subpopulation of Nestin-positive cells and cells expressing leptin receptor (LepR) throughout the bone marrow (Kunisaki et al. 2013). While $LepR^{+}$ cells are indeed a major source of osteogenic and chondrogenic cells throughout adulthood (Zhou et al. 2014) and share an overlap with Nestin-positive cells, this can in part be attributed to the limitations related to the Nestin-GFP label. Moreover, over 17 different stromal subpopulations express LepR (Baryawno et al. 2019), less than 10% of which is capable of colony formation (Zhou et al. 2014). These labels are therefore not markers for *bona fide* SSCs (Zhou et al. 2014).

Gremlin-Cre traced cells (Worthley et al. 2015) located in the murine metaphysis have demonstrated all hallmarks of a true SSC. Furthermore, neither Nestin-Cre nor LepR-Cre are capable of tracing these $Grem1^{+}$ cells. This would indicate that these cells are true SSCs, found at the boundary of the growth plate. The growth plate itself is indeed a reservoir of stem cells, where at least two more stem cell populations are driving the clonal expansion that keeps bones growing (Mizuhashi et al. 2018) (Newton et al. 2019). A fourth stem cell population at the growth plate is marked by Glioma-associated oncogene 1 (Gli1) and appears crucial for the maintenance of bone mass in postnatal life (Shi et al. 2017).

Chan *et al.* defined a human SSC population which is hematopoietic lineage negative, does not express the pericyte marker CD146, but is positive for CD164, podoplanin (PDPN), and CD73 (Chan et al. 2018). The nonoverlap can be explained by the ability of the pericyte-like cell to give rise to fat and its subendothelial localization. In addition, a bone, cartilage, and stroma precursor derived from Chans SSCs is $CD146^{+}$. In previous work, Chan *et al.* had identified a mouse SSC, which is $CD45^{-}Ter119^{-}Tie2^{-}AlphaV^{+}Thy^{-}6C3^{-}CD105^{-}CD200^{+}$ (Chan et al. 2015). While the cell surface markers do not appear to correlate well between mouse and human, the identification was performed based on transcriptome similarity, measured through single-cell RNA sequencing. Both SSCs were discovered in the growth plate, but the hSSC could also be isolated from the periosteum, the adult femoral head, and the fracture callus. In addition, hSSC-like cells can be generated from iPSCs or BMP2-treated human adipose stroma (HAS).

While of great importance for our knowledge of bone in growth and homeostasis, periosteal stem cells are of particular interest as a cell source for clinical application in a tissue engineering context. It is widely accepted that the SSC responding to fracture is located in the periosteum (Marecic et al. 2015). Colnot and colleagues described a multipotent periosteal stem cell (PSC) dependent on the presence of the matrix protein Periostin (Duchamp de Lageneste et al. 2018) which gives rise to most cells in the fracture callus. Another periosteal stem cell upstream of the LepR-Cre and Osx-Cre stromal cell populations is marked by expression of HoxA11 (Pineault et al. 2019). A third PSC is marked by expression of Mx1 and α-smooth muscle actin (α-SMA). These cells and their progeny gave rise to most of the fracture callus and were also found to express CD140 and Grem1, but not Nestin (Ortinau et al. 2019).

A final periosteum-specific SSC and two distinct periosteal progenitor populations were identified by Debnath et al., using a green fluorescent protein (GFP) cathepsin K reporter mouse (Debnath et al. 2018). All populations were negative for hematopoietic lineage markers, did not express CD90.2 and 6C3, and had low expression of CD49f and CD51. The periosteal stem cell (PSC) was defined $CD105^{-}CD200^{+}$, while the progenitor populations were $CD105^{-}CD200^{-}$ and $CD105^{+}CD200^{var}$. A human equivalent of the PSC was also identified by the $CD105^{-}CD200^{+}$ signature. These stem and progenitor cells were found to exclusively perform intramembranous ossification during homeostasis, but acquired osteochondrogenic differentiation potential during fracture healing. This plasticity is well in line with the role of the periosteum in development and repair.

Taken together, a plethora of SSCs has been described across all skeletal compartments including the periosteum, summarized in Table 1 and recently reviewed (Ambrosi et al. 2019). The extent to which these cells are truly distinct subpopulations and not a common population described by different markers in various settings and models remains to be addressed. Another issue is the limited work performed in man. However, in the context of fracture healing, it can be concluded that the periosteum appears a very relevant source of stem and progenitor cells.

6 Isolation of Periosteum-Derived Cells

Human periosteum-derived cells (hPDCs) can be isolated from patients undergoing surgery for bone fracture repair without the need of additional surgery. Of note, the periosteum becomes gradually thinner with age and is nearly invisible in patients of high age, reducing both the number of cells to isolate, but also the osteochondrogenic potential (Moore et al. 2014). The quality of the samples and the number of cells possible to isolate is therefore highly dependent on the age of the patient, with minors or adolescents being the ideal donors. Generally, periosteal biopsies are small (~0.5 cm^2) as to not jeopardize a positive clinical outcome and approximately 4000 plastic adherent cells can be isolated/mg of periosteal tissue after the first passaging (Fig. 3a). Since most layers of the periosteum are generally cell-poor, it is highly advised to optimize the cell isolation procedure for the specific setting. Primary culture of hPDCs therefore usually starts out in a 6-well plate, as hPDCs

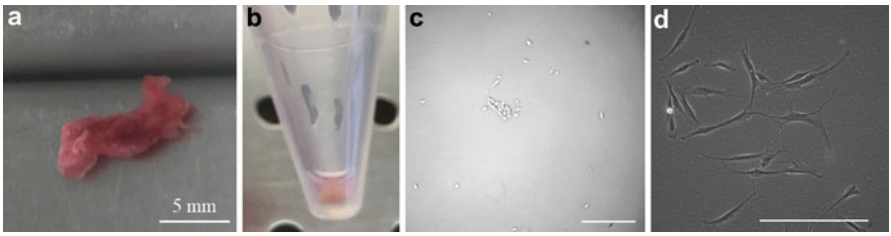


Fig. 3 Steps of PDC isolation. A typical sample of human periosteum as obtained from patients undergoing osteosynthesis or distraction osteogenesis (**a**). After digestion, the pellet should be clear of RBCs (**b**). Approximately 12 h after seeding, nonadherent cells are removed. PDCs typically have an elongated fibroblast-like phenotype, show at 4X (**c**) and 10X (**d**) magnification. Scale bar: 200 μm.

tend to slow down their proliferation speed at low confluency. Mouse periosteum-derived cells (mPDCs) are slightly easier to obtain, since after sacrifice of the mice, periosteum from entire long bones (e.g., femur, tibia, fibula) can be stripped to collect cells. The processing of periosteum from tissue to single cell is species agnostic and described below.

A combination of mechanical and enzymatic dissociation of the tissue is optimal for maximum cell yields. The mechanical step usually involves the cutting of the periosteum into the smallest possible pieces (~ 0.5 mm) with a scalpel or razor blade, which optimizes the surface area exposed to the proteolytic enzymes. The periosteum is significantly easier to cut after removal of excessive liquid by gently placing on a sterile gauze pad.

Regarding enzymatic digestion, multiple options are available. Liberase has been used (He et al. 2017b), although a combination of collagenase and dispase may be preferable. Moreover, both type II and type IV collagenase can be used to isolate hPDCs and mPDCs. Collagenases are proteases which cleave Pro-X-Gly-Pro sequences between X and glycine, where X is a neutral amino acid. This sequence is found with high frequency in collagen. Collagenase is unique among proteases in its ability to degrade the triple-helical native collagen fibrils.

Often, collagenase solutions are mixed with dispase to further enhance the breakdown of the matrix. Dispase is a gentle protease which cleaves fibronectin, collagen type IV, and collagen type I, all of which are present at high levels in the periosteal matrix.

After enzymatic digestion, the duration of which should be optimized, the collagenase-dispase mix is removed by centrifugation, and the cell pellet is resuspended in culture medium (Fig. 3b). It is often not possible to perform a cell count for hPDCs at this point due to their low numbers. Instead, seeded hPDCs should be viewed through a brightfield microscope and cell attachment can be visualized just hours postseeding (Fig. 3c). Within 2–4 h after seeding, most hPDCs adhere to the plastic of the culture plate and begin to display their characteristic spindle-like elongated morphology (Fig. 3d).

In the event where large pieces of debris or cell aggregates are observed, multiple options are available for their removal. Visibly large debris can be removed by passing the lysate through a 70-μm nylon mesh. Aggregates of cells can be dissociated by thoroughly and carefully pipetting the cell suspension with a 1000-μl pipette. This step is gentle on the cells, but should only be performed in the absence of debris, to prevent the clogging of the pipette tip. If unsuccessful, deoxyribonuclease I (DNase I) represents a gentle option. DNA released by apoptotic or dead cells increases the viscosity of the sample and can lead to the aggregation of cells. In cases where high numbers of cell clumps and dead cells are observed, DNase is the recommended next step to try and increase the single cell yield.

As previously mentioned, the periosteum is highly vascularized. It is therefore very well possible that a large amount of cells from the hematopoietic lineage contaminate the sample. The most straightforward way to remove these cells is to wait for the PDCs to adhere and change the medium which contains the plastic nonadherent blood cells. This should be done within 24–48 h after the start of the culture. If left unchecked, red blood cells (RBCs) will die, which releases reactive oxygen species (ROS) into the culture. NK cells can also have detrimental effects on the culture, as their cytotoxicity is regulated by a balance of stimulatory and inhibitory signals. The culture medium does not support this tightly regulated balance, which therefore results in cytotoxicity towards the hPDCs.

6.1 In Vitro Expansion

Tissue engineered living implants require large numbers of cells, estimated from 15 to 45 million cells for restoring the joint surface, to over 500 million to bridge a 4 cm tibia defect (Wakitani et al. 2002) Traditionally, PDCs are expanded in 2D cultures in media supplemented with fetal bovine serum (FBS). Under serum-containing conditions, hPDCs have shown a remarkable *in vitro* expansion potential for up to 30 population doublings with a doubling time of approximately 55 hours (Lambrechts et al. 2016). While FBS provides good results in terms of expansion, it has a few drawbacks. First and foremost, it is xenogeneic, which hampers translation to the clinic. Secondly there is major batch-to-batch variability in the composition of FBS. In addition, FBS contains unknown concentrations of numerous biologically active components which are known to interfere with periosteal chondrogenesis, TGF-β1 and BMP signaling (Fitzsimmons et al. 2004; Shahdadfar et al. 2005). In recent years, human platelet lysate (hPL) has been suggested as an alternative medium supplement (Astori et al. 2016; Doucet et al. 2005). While hPL also has lot-to-lot variability, it has the advantage of being xeno-free. Pooling of large numbers of donors (20–120) is now being performed to reduce this batch effect (Strunk et al. 2018). One of the most striking differences which can be observed when comparatively culturing hPDCs in FBS- and hPL-supplemented media is the increased proliferation. This observation is consistent for multiple sources of cells originating from the mesenchyme, including the bone marrow, umbilical cord, corneal stroma, and adipose tissue (Ben Azouna et al. 2012; Fazzina et al. 2016;

Matthyssen et al. 2017; Reinisch et al. 2007). In addition, hPL-expanded MSCs were reported to have an enhanced osteogenic and chondrogenic differentiation potential (Jonsdottir-Buch et al. 2013; Salvade et al. 2010). To address the issues, with interfering ligands, stimulatory factors, and batch-batch inconsistency, a shift toward alternate supplements and chemically defined media (CDM) has been advocated and intensively investigated (Bolander et al. 2017, 2019).

It is important to stress that PDC culture conditions need to be optimized depending on the source of the periosteum. This entails the basal media, the growth factor-containing excipients, and combinations thereof. Human- and sheep-derived PDCs can be efficiently expanded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. For hPDCs, this 10% FBS can be replaced by 10% hPL, with positive effects on their proliferation and bone-formation capacity (Gupta et al. 2019). Sheep PDCs on the other hand also proliferate faster when cultured with 10% hPL, but also rapidly become senescent (in-house data, unpublished). DMEM as a basal medium does not support expansion of murine PDCs, where α-minimal essential medium is advised as the basal medium of choice (van Gastel et al. 2012).

6.2 3D Culture

Bioreactor setups have been proposed to increase the scalability of cell therapies. One option for 3D bioreactor culture is the use of spinner flasks with microcarriers. Using collagen-based microcarriers, it was shown that a spinner flask setup with hPL-supplemented medium had multiple advantages: hPDC proliferation speed increased dramatically, while improving the *in vivo* bone forming capacity, which is a critical parameter in bone tissue engineering. The significant decrease in culture time due to the use of hPL and a 3D-setup is likely to make this strategy more cost-effective in the long run (Gupta et al. 2019).

7 Periosteum Derived Cells in Regenerative Medicine

In the development of a successful cell-based implant for a critical bone fracture, the first step is to make a clinical characterization of the defect (Fig. 4a). This includes to define the size, stability, complexity (which tissue types are affected), severity of damaged surrounding soft tissue, potential vascularization issues, and identification of the required treatment strategy. This may help to define which regenerative route (e.g., intramembranous or endochondral fracture healing) may be required.

The next step is to define how the implant should be designed to meet critical regulatory and clinical criteria. In critical sized defects or defects with a severely damaged surrounding environment, a cell-based implant may significantly improve the potential timely success of regeneration. Next, biologists can determine which crucial biological processes that are involved in the tissues to be regenerated and define which factors and molecular events are required for the recapitulation thereof based on a the appropriate choice of progenitor cell (Fig. 4b). With a cell-based

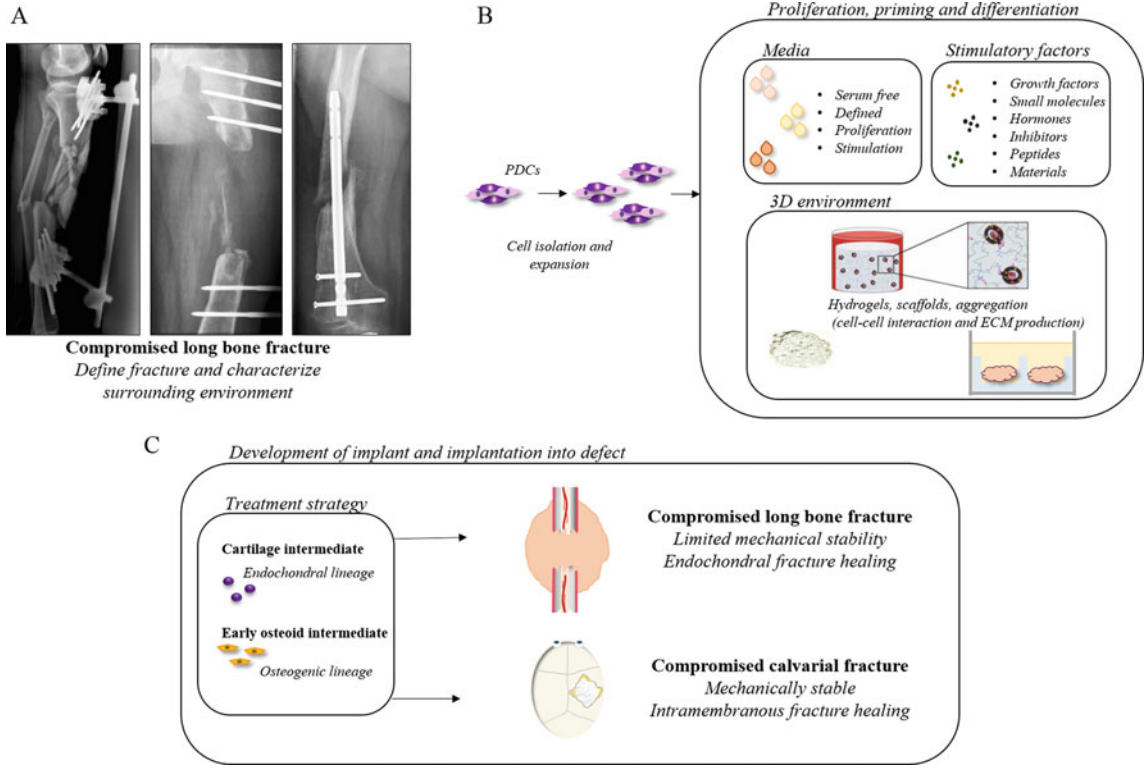


Fig. 4 Steps to take into consideration in the development of a cell-based construct for the treatment of critical bone fractures. (**a**) Clinical characterization of the defect in terms of location, size, damages tissue(s), affected surrounding environment, co-morbidities. (**b**) Based on the characterization,

implant, there are often a critical number of cells that are required to induce differentiation. Therefore, to reach sufficient cell numbers, cell expansion can be carried out according to standard protocols *in vitro*, or postimplantation *in situ*. If the latter is chosen, the issues with integration will be limited, but require a sophisticated environment that also induces sufficient steering for differentiation and matrix production. The next step is differentiation strategy, which molecules and 3D environment are required to steer the specific path of differentiation. As a final step, cell and tissue maturation needs to be directed *in vitro* and the final living tissue intermediates are then assembled for implantation into the fracture site (Fig. 4c). Uniquely, periosteum derived cells are crucial, required, and actively contributing to both intramembranous and endochondral fracture healing and their potential is ideal for the development of skeletal tissue regenerative constructs for the treatment of both direct and endochondral tissue regeneration.

8 Periosteum Derived Cells for Intramembranous Fracture Healing

The cells provide the driving force in a cell-based construct for regenerative purposes, but their differentiation and matrix secreting potential is stringently depending on the presented micro- and macroenvironment. Due to the complex environment that is represented by the progenitor cells homing niche *in vivo*, it is a difficult task to create an *in vitro* equivalent. Consequently, extensive research in terms of development of scaffolds and stimulatory molecules regarding how these affect cell behavior has been carried out. Initially, a scaffold was mainly used for delivering cells to the defect site, but as our understanding of the crucial role of the microenvironment on cell behavior evolved, the surrounding matrix is today almost as important as the cell itself. Scaffolds can be made of natural or synthetic biomaterials. Appropriate carriers for skeletal tissue regeneration, and in particular for long bone fractures should possess osteoconductive and/or osteoinductive properties, while supporting osseointegration. For intramembranous bone formation, Ca^{2+}-based scaffolds have shown to be suitable carriers for periosteum derived cells. In an early *in vivo* ectopic study in mice, Eyckmans *et al.* showed that when *in vitro* expanded human periosteum derived cells (hPDCs) seeded on a Collagraft™ carrier (an open porous composite made of CaP granules consisting of 65% hydroxyapatite (HA) and 35% β- tri-calcium phosphate (β-TCP), embedded in a bovine collagen type I matrix), were able to form ectopic bone 8 weeks postimplantation (Eyckmans et al. 2010). In order to understand the osteoinductive effect of CaP-based materials on hPDCs, the

Fig. 4 (continued) the regenerative processes required to treat the defect successfully need to be identified to design a suitable cell-based implant in terms of cell population to use, cell expansion, what medium is needed, proliferative and differentiation-inducing factors, and what kind of 3D environment is favorable? (**c**) Then these factors are combined in the development of a cell-based construct for the treatment of the critical defect

mechanisms of action were investigated. It was shown that the *de novo* bone was mainly formed by the hPDCs, and the process required Ca^{2+} in the scaffold to initiate the osteoinductive program in the implanted cells. Moreover, inhibition of endogenous bone morphogenetic protein and Wnt signaling by overexpression of the secreted antagonists Noggin and Frzb, respectively, also abrogated osteoinduction. Further, it was shown that proliferation of the engrafted hPDCs was strongly reduced in the decalcified scaffolds or when seeded with adenovirus-Noggin/Frzb transduced hPDCs. This indicated that cell division/proliferation of the engrafted hPDCs was required for direct bone formation. It can be hypothesized that this would not be required with higher cell seeding numbers. However, higher seeding densities did not improve bone formation, implicating that higher cell numbers may limit availability of nutrients thus jeopardize the survival of implanted cells. Lower seeding number on the other hand allows the cell to proliferate to reach the required cell density in the implant, prior to the onset of differentiation.

Subsequently, enhanced *in vitro* differentiation prior to *in vivo* implantation of the same progenitor cell population was studied to improve bone formation. For this, hPDCs were seeded on Collagraft™ scaffolds (calcium phosphate rich matrix or CPRM) or on decalcified scaffolds (calcium phosphate depleted matrix or CPDM), followed by subcutaneous implantation in nude mice to trigger ectopic bone formation. In this system, CPRM, but not CPDM scaffolds that lacked Ca^{2+}, supported osteoblast differentiation and bone formation by the seeded hPDCs. Microarray gene expression analysis at 20 h after seeding, and 2, 8, and 18 days after implantation showed that both matrices triggered a similar gene expression cascade. However, gene expression dynamics progressed faster in CPRM scaffolds compared to CPDM scaffolds. The difference in transcriptional dynamics was associated with differential activation of hub genes and molecular signaling pathways related to calcium signaling (CREB), inflammation (TNFα, NF-kB, and IL-6), and bone development (TGFβ, β-catenin, BMP, EGF, and ERK signaling). Starting from this set of pathways, a growth factor cocktail was developed that could enhance osteogenic differentiation *in vitro* and *in vivo* bone formation through the intramembranous pathway. With the key factors required for CaP-induced bone formation by hPDCs identified, the effect of the specific characteristics of the CaP-material was assessed next. CaP naturally exists in bone as hydroxyapatite, but can also be used as a synthetic or partly synthetic material. Interestingly, it was shown that scaffolds containing natural hydroxyapaptite were more beneficial for bone formation, compared to fully synthetic materials in combination with hPDCs (Roberts et al. 2011).

The unknown mechanism by which natural biomaterials showed improved osteoinductive properties when seeded with hPDCs in comparison with synthetic materials has hampered the optimization of novel biomaterials suitable for the clinical setting. It was recently shown that this may be due to a different activation of the innate immune system (Sadtler et al. 2019). Therefore, further knowledge regarding how these different materials activate the early molecular events governing bone tissue formation by hPDCs was required. For this, hPDCs were combined with three types of clinically used CaP-scaffolds to obtain constructs with a distinct (high-natural HA), moderate (partly natural HA), and no (synthetic HA)

bone forming capacity in combination with hPDCs *in vivo*. Protein phosphorylation together with mRNA transcript analysis for key ligands and target genes was investigated 24 hours *post*-cell seeding *in vitro* and 3 and 12 days *post*-ectopic implantation in nude mice. A computational modeling approach was used to deduce critical factors and the level of activation that was required for bone formation 8 weeks *post*implantation. Interestingly, the combined Ca^{2+}-mediated activation of BMP-, Wnt-, and PKC signaling pathways 3 days *post* implantation was able to discriminate the bone forming from the nonbone forming constructs. Subsequently, a mathematical model able to predict *in vivo* bone formation by hPDCs with 96% accuracy was developed. These results illustrated the importance of defining and understanding key CaP-activated signaling pathways required for *in vivo* bone formation. With this information, it was clear that hPDCs can be robustly steered towards *in vivo* intramembranous bone formation when seeded onto CaP-based carriers with appropriate characteristics. However, the intramembranous bone forming process is slow and routinely only generates limited amounts of bone and bone marrow by the implanted cells. In a complex environment such as a large fracture, it is crucial to get a bridging between the bone ends for successful healing. Consequently, the endochondral route may be of greater value as a target for the typical complex bone fractures requiring treatment in the form of a cell-based construct.

9 Periosteum Derived Cells for Endochondral Fracture Healing

Periosteum derived cells have been shown to be crucial in long bone fracture healing due to their contribution to both the intramembranous and endochondral bone healing process. Furthermore, their activation is to a large extent initiated and steered by members of the BMP-family (Salazar et al. 2019; Tsuji et al. 2006). In an attempt to enhance and steer the bone forming capacity of hPDC-CaP-constructs towards the endochondral pathway, the osteochondrogenic response of BMP-ligands with a known role in fracture repair has been evaluated *in vitro (*Bolander et al. 2016*)*. BMP-2, -4, -6, and -9 were shown to induce *in vitro* differentiation of hPDCs. Subsequently, these ligands were coated onto clinically approved CaP-scaffolds with limited (BioOss®) or no (CopiOs®) bone forming capacity followed by seeding with hPDCs. Protein lysates and conditioned media were investigated for activation of BMP signaling pathways 24 h *post* seeding. Upon *in vivo* implantation, the most abundant bone formation was found in BMP-2 and BMP-6-coated scaffolds 8 weeks *post*implantation. Interestingly, implanted cells actively contributed to the newly formed bone and remnants of cartilage could be observed in BMP-coated CopiOs®-constructs. This confirmed the synergistic effect of combined CaP and BMP stimulation for bone formation by hPDCs. Interestingly, computational analysis displayed that the type of BMP-ligand as well as the specific CaP-scaffold affects skeletal tissue formation, observed in a qualitative as well as quantitative manner (Bolander et al. 2016).

From the BMP-family members, BMP-2 and BMP-7 were the first to obtain approval for clinical applications. However, their use has been associated with unpredictable bone formation. This has been attributed to a number of factors including the delivery of supraphysiological levels of BMPs and insufficient progenitor cells in the damaged fracture environment (Pobloth et al. 2015; Shields et al. 2006). *In vitro* primed cell-based regenerative constructs could overcome this hurdle by delivering the crucial number of appropriately primed cells *in vitro* to regenerate the damaged tissue *in vivo*. However, *in vitro* stimulation of progenitor cells for *in vivo* tissue regeneration is challenging. Long-term *in vitro* stimulated cells often fail to integrate with the host, likely due to the maturity of the tissue (Yamashita et al. 2015). Shorter priming periods on the other hand do not seem to be sufficient in terms of clinically relevant results (Eyckmans et al. 2013). A potential reason for this can be that the *in vitro* priming is carried out in media containing serum (Ryan 1979). Indeed, serum contains an undefined and variable range of factors such as cytokines and inhibitors that bind to cell surface receptors. In consequence, batch-to-batch variability significantly affects the characteristics of cell-based implants, leading to unpredictable behavior and outcomes (Baker 2016; Jung et al. 2012). Since stimulatory ligands bind to cell surface receptors which may be occupied by factors in the serum, a serum free preconditioning of hPDCs prior to growth factor treatment was hypothesized to improve the cellular response. Consequently, a preconditioning regime of hPDCs in serum free chemically defined media (CDM) was developed which led to adapted progenitor cell subpopulations with improved osteochondrogenic differentiation capacity (Bolander et al. 2017). This phenotype shift was marked by reduced positivity for CD105 but enhanced positivity for CD34 together with elevated mRNA transcript expression of marker genes such as FGF2, VEGF, and BMP type 1 and 2 receptors. The preconditioned hPDCs was then assembled into microspheroids in order to mimic the cellular condensations preceding endochondral fracture healing and by providing biomimetic 3D cues. The microsperoids were simultaneously treated with BMP-2 under serum free conditions, and cell specification towards the osteochondrogenic lineage was observed *in vitro* (Bolander et al. 2017). Interestingly, physiologically relevant levels of autocrine and paracrine growth factors were secreted by the fate-steered engineered microtissues. *In vivo*, the self-sustained implant proceeded to form bone in the ectopic setting and was able to regenerate a critical size long bone defect in mice through the endochondral pathway. Interestingly, the implanted cells largely contributed to the formation of the initial cartilaginous callus at week 4 and to the immature bone at week 4, but less to the remodeled mature bone at week 8. These findings suggest that the major impact of the implanted cells occurs in the early phase and aids in the recruitment of cells from the host environment that subsequently aid in the later stage remodeling phase.

In a similar approach, periosteum-based microspheroids were used for the scalable production of a larger implant, developed through the assembly by fusion between the microspheroids after 7, 14, 21, or 28 days of *in vitro* differentiation in a growth factor cocktail consisting of standard low glucose growth medium supplemented with ascorbate-2 phosphate, dexamethasone, proline, Rho-kinase inhibitor Y27632, ITS+, BMP-2, GDF5, TGF-β1, BMP-6, and basic FGF-2 (Nilsson

Hall et al. 2020). It was shown that a significant maturation of the microspheroids took place between day 14 and 21, reflected by elevated expression of FOXA2, DMP1, and SCIN which are crucial for chondrocyte hypertrophy (Ionescu et al. 2012), cartilage–bone transition (Ye et al. 2005), and bone resorption (Song et al. 2015), respectively. *In vivo*, histology and immunohistochemistry was used to distinguish between the day 14 and day 21 samples as "early prehypertrophic" stage for day 14, and "late prehypertrophic" stage for day 21 modules. Based on these findings, microspheroids from day 21 were used as microtissues to fuse in a mold design to mimic the critical size defect in a nude mouse model. Upon implantation, the macrostructure was able to heal the defect through endochondral bone formation. The regenerated and remodeled bone exhibited already after 8 weeks similar morphological properties to those of native tibia. The significance of these research findings lies in the ability to use the microspheroids as a living "bio-ink" allowing bottom-up manufacturing of multimodular tissues with complex geometric features and specified quality attributes.

In an attempt to further understand the improved regenerative potential by serum free preconditioning of hPDCs, in depth profiling by single-cell RNA sequencing was performed (Bolander et al. 2019). Interestingly, preconditioning in CDM was shown to induce a phenotype switch at the single-cell level with elevated expression of markers and signaling clusters associated with skeletal system development, tissue regeneration, stem cell maintenance, cell fate commitment, and the BMP-signaling pathway. In a comparative analysis between cells in serum-containing growth medium (GM) and CDM by clustering of the complete data set, it was shown that the individual clusters from CDM origin displayed elevated markers and processes associated with native osteochondral progenitor cells. On the other hand, the majority of the GM-originated clusters were more heterogeneous, with subpopulations enriched for markers related to connective tissue as well as chondrogenic and osteogenic progenitors. These findings once again confirmed the more homogenous and osteochondro-specific progenitor commitment of the CDM subpopulation. Detailed analysis on the inferred transcription factor activity linked upregulated and active involvement of the SOX4, SOX9, MSX1, and RUNX2 regulons with the enhanced bone forming potential in the CDM population. Of note, these data were in line with recent findings mapping the hierarchy of human skeletal stem and progenitor cells present in the human fetus, but also activated during postnatal fracture repair (Chan et al. 2018). Specifically, it is of great importance that a similar progenitor cell program can be activated after extensive *in vitro* expansion, a phenomenon known to reduce the progenitor cell potential in the presence of serum.

Furthermore, when the serum-free preconditioned cells were primed with BMP-2 under serum-free conditions for an additional 6 days *in vitro*, and then seeded onto the CopiOs® carrier followed by *in vivo* implantation, enhanced bone formation was seen in comparison to cells cultured under serum-containing conditions. In order to define a marker for the cell population with enhanced bone forming capacity, BMP-receptor expression was investigated on the protein level due to the indispensable role of BMP-ligands as stimulatory factors in the periosteum during fracture healing (Salazar et al. 2019). BMP-ligands signal through a complex of type

1 (ALK1/ACVRL1, ALK2/ACVR1, ALK3/BMPR1A, ALK6/BMPR1B) and type 2 (BMPR2, ACVR2A, and ACVR2B) transmembrane serine/threonine kinase receptors. As such, the expression level and availability of the BMP-receptors on a progenitor cell are of relevance for their ability to respond and undergo subsequent differentiation upon BMP-stimulation (Salazar et al. 2016). Unfortunately, this part is an often neglected parameter in the preparation and characterization of cell-based constructs. Instead, current progenitor markers are selected based on the cell's ability to proliferate and differentiate under nonphysiological conditions (International Stem Cell Initiative et al. 2010; Verbeeck et al. 2019).

Initially, the expression of BMP-receptors was investigated by mRNA transcript analysis and on the protein level by flow cytometry. Encouragingly, all investigated receptors including ALK2, ALK3, ALK6 and BMPR2 were confirmed to be upregulated upon serum-free preconditioning both in terms of number of positive cells, but also in number of receptors per cell. However, a specifically drastic increase was seen for BMPR2, which was therefore selected for further investigation. When sorting for $BMPR2^+$ cells, elevated bone forming capacity was confirmed even with reduced cell numbers. In addition, silencing of BMPR2 abrogated fracture healing in an orthotopic mouse model for a critical size defect. Interestingly, the improved cell differentiation seen in the serum-free preconditioned hPDCs was associated with a cellular switch towards a more efficient metabolism, potentially related to the elevated resistance to harsh conditions as encountered during *in vivo* implantation. These findings support the importance of the appropriate design and development of cell-based constructs.

In terms of biomaterial for endochondral tissue formation by hPDCs, CaP-based materials have shown to be suitable in combination with BMPs. In addition, polyethylene glycol (PEG) hydrogels functionalized with the cell-binding motif Arginine-Glycine-Aspartic Acid (RGD) have been confirmed to support proliferation, chondrogenic gene expression, and matrix production of encapsulated hPDCs (Kudva et al. 2018). In growth medium, the hPDCs in the RGD-functionalized hydrogels maintained high levels of viability and demonstrated an enhanced proliferation when compared with hPDCs in nonfunctionalized hydrogels. Additionally, the RGD-containing hydrogels promoted higher glycosaminoglycan (GAG) synthesis and chondrogenic gene expression of the encapsulated hPDCs, as opposed to the nonfunctionalized constructs, when cultured in two different chondrogenic media. These results demonstrated the potential of hPDCs in combination with enzymatically degradable PEG hydrogels functionalized with adhesion ligands for cartilage regenerative applications. Interestingly, the chondrogenic phenotype in the encapsulated hPDCs could be further enhanced upon the addition of TGF-β releasing beads (Kudva et al. 2019).

10 Osteochondral Tissue Repair by Periosteum Derived Cells

Human periosteum derived cells have also been used for osteochondral tissue repair. *In vitro* expanded periosteal cells were seeded in micromass cultures and simultaneously stimulated with a growth factor cocktail comprising transforming growth

factor (TGF)-β1, BMP-2, growth differentiation factor (GDF)5, BMP6, and fibroblast growth factor (FGF)2 for 28 days (Mendes et al. 2018). To evaluate *in vivo* tissue formation by the *in vitro* treated micromass cultures, they were implanted ectopically in nude mice and orthotopically in critical-size osteochondral defects in nude rats followed by evaluation through microcomputed tomography (μCT) and immunohistochemistry. mRNA transcript analysis after 28 days of *in vitro* culture revealed the expression of early and late chondrogenic markers and a significant upregulation of NOGGIN as compared to human articular chondrocytes. Histological examination revealed a bilayered structure comprising of chondrocytes at different stages of maturity. Ectopically, the implanted tissues generated both bone and mineralized cartilage at 8 weeks *post* implantation. Osteochondral defects treated with the *in vitro* stimulated cells displayed glycosaminoglycan (GAG) production, type-II collagen, and lubricin expression. Immunostaining for human nuclei suggested that hPDCs contributed to the repair of both the subchondral bone and articular cartilage. This study was the first to use periosteal cells for stable cartilage formation. Due to their fate towards the osteogenic and endochondral lineage, it is hypothesized that PDCs would not be able to form articular cartilage and it is not yet clear why the *in vitro* primed tissues formed bone in the ectopic setting, while it formed articular cartilage when implanted in the osteochondral defect in the joint. This suggests that the differentiation events depend on their specific location and thus environmental cues. However, it remains to be evaluated how these implants perform in a larger animal model with a more clinically relevant load, as well as in a long term study.

11 Periosteal Cells in the Preclinical Setting

Rodents represent the most commonly utilized models to evaluate the *in vivo* behavior of *in vitro* engineered cell-based implants. Even though they fill an important purpose to deliver Proof of Concept as a screening model, obtained results are largely biased since the small scale generally avoids issues with nutrient depletion and upscaling. Therefore, larger animals are required prior to clinical translation and a summary of some reported models and their advantages and disadvantages are presented in Table 2.

In general, larger animal models are solely used as orthotopic models and can from a surgical point of view in the field of skeletal tissue engineering be categorized as (1) calvarial defect, (2) segmental long bone defect, and (3) osteochondral defect, all three with different critical size highly depending on location, age of animal and species. The calvarial defect provides a good nonloading bone healing environment with relative biological inertness due to poor blood supply and limited access of bone marrow. This is essential since the model is aimed to resemble the atrophic mandibular/craniofacial bone in humans. Furthermore, it provides a suitable large scale environment to study the design of implants to follow the intramembranous ossification route. The standard rodent calvarial bone defect is typically created by using a trephine drill that makes a circular defect in the cranial skeleton on the

Table 2 Overview of large animal models for cell-cased implants targeted for skeletal tissue engineering

Animal	Advantages	Disadvantages	Calvarial defect	Segmental long bone defect	Osteochondral defect	Reference
Rodents (mice & rat)	Easy to perform surgery Availability of Immunodeficient animals Availability of specific disease-target geneknock out animal.	Relatively thicker and fewer trabeculae than humans Mice and rat do not have Haversian system Periosteum in rats and is well-vascularized, hence improving bone healing.	Mice: 5 mm diameter Rat: 8 mm diameter	Mice: 0.4 cm in radius, 0.5 cm in femur Rat: 1 cm in radius, 0.4–0.5 cm in tibia, 0.5–1 cm in femur.	Mice: Rat: 1 mm depth, 1.4 mm I diameter	(Barak et al. 2013; van Griensven 2015; Vashishth 2008) (Cooper et al. 2010)
Rabbit	Easy to handle and small size, reaching skeletal maturity shortly after sexual maturity at ~6 m of age. Similarities with human bone are reported in bone mineral density and subsequently the fracture toughness of mid-diaphysea	Small size; differences in bone anatomy, such as size and shape of the bones and also in loading; faster skeletal change and bone turnover the rabbit is a vulnerable animal prone to post-antibiotic diarrhea, gastrointestinal stasis or ileus, and postimplantation fractures due to a fragile weight-bearing femur	10 mm in diameter	The mid-femur diameter is only about 5 mm, tibiae defect: 20 mm	4 mm depth, 4 mm in diameter	(Bakker et al. 2008; Berninger et al. 2013; Castaneda et al. 2006; Ito et al. 2005; Mapara et al. 2012; Struillou et al. 2010; Wang et al. 1998; Xu et al. 2008)

(continued)

Table 2 (continued)

Animal	Advantages	Disadvantages	Calvarial defect	Segmental long bone defect	Osteochondral defect	Reference
Sheep	Docile animals with easy outdoor housing Similar body weight to humans Hind-limb anatomy similar to humans Dimension of long bones suitable for human implants	Higher trabecular bone density than humans Late skeletal maturity, with Haversian remodeling at 7–9 years of age	30 mm in diameter, unspecified depth but intact dura mater reported	30–35 mm in tibia 25 mm in femur	7 mm < full thickness	(Blaszczyk et al. 2018; Horner et al. 2010; Schinhan et al. 2012; van Griensven 2015; Yu et al. 2013)
Pig	Better social acceptance Bone mineral density and healing similar to humans	Hind-limb anatomy is different to humans Rapid growth rate Difficult handling	10 mm diameter, 10 mm depth	25–30 mm in radius	10-mm diameter, 4-mm depth	(He et al. 2017a; Horner et al. 2010; van Griensven 2015; Wehrhan et al. 2013; Yu et al. 2013)

midline (Szpalski et al. 2010). However, the reliability of the model is highly depending on the precision of the sagittal suture and careful protection of the dura mater underlying the defect, important for the healing of the cranial skeleton. Furthermore, the filling materials should be strong and resistant enough to avoid the dilation of brain tissue beneath the defect (Ji et al. 2012). The use of periosteal cells for the treatment of a calvarial defect in a porcine model was utilized in a study with the goal to investigate the bone forming potential of progenitor cells from adipose, bone marrow, and periosteal tissue (Stockmann et al. 2012). Autologous progenitor cells were harvested and expanded *in vitro* to reach sufficient cell numbers. Thereafter, cells were seeded onto collagen scaffolds of low cross-linked bovine collagen type I and cultured for osteogenic differentiation in the presence of osteogenic induction medium for another 7 days. Then, a coronal-sagittal approach was used in the forehead region of the pig and cylindrical defects of 1 cm in depth and 1 cm in diameter were created. The internal plate of the neurocranium remained completely intact during the procedure and the cell-seeded scaffolds were subsequently implanted into the fresh monocortical calvarial bone defects. At 90 days, the majority of defects treated with autologous cells showed complete osseous regeneration but the control defects without implanted cells did not demonstrate bone healing. Off note, no difference in bone regeneration induced by the different cell population was noted (Stockmann et al. 2012).

Segmental long bone defects on the other hand allow researchers to test and understand the regenerative implants destined for long bones. The creation of segmental long bone defects is usually performed through an osteotomy approach utilizing a drill or saw to surgically remove the required length of bone from a predetermined site, producing a consistent defect in all subjects. The bone itself can be fixed internally with either bone plates, intramedullary rods (Horner et al. 2010) or by an external fixator such as the Ilizarov fixation technique (Lammens et al. 1998). Equally important as fixation is the specific segmental model in terms of defect environment. Bone regeneration is an area where biology is directly correlated with geometric and mechanical conditions, and combined play an indisputable role which needs to be taken into account in translational research. The segmental bone defect models is often used to evaluate implants developed for the treatment of nonunion bone fractures, representing a highly avascular environment filled with fibrotic tissue. Therefore, it is important that not only the defect size, but also the surrounding environment represent a clinically relevant setting. Since this is often a neglected factor, clinical translation remains a considerable challenge, in particular since robust outcomes in well-defined large animal models are lacking. In an attempt to develop a reliable model of a nonunion defect (pseudoarthrosis), skeletal immature and mature sheep were evaluated with tibial bone defects of both 3.0 and 4.5 cm in immature and 4.5 cm in mature animals (Lammens et al. 2017). The size of the defects was selected based on the guidelines that critical size defects should be at least 2 to 2.5 times the diameter of the bone. According to these guidelines, a 3 cm defect should be made in the skeletal immature sheep and a 4.5 cm defect in the skeletal mature. Upon defect creation, gaps were left empty during a period of 6 weeks to allow ingrowth of fibrotic scar tissue that was subsequently removed

for the implantation of a cement spacer for another 6 weeks to induce a Masquelet membrane (Masquelet and Begue 2010). After the 6 weeks of membrane induction, 6/12 of the young sheep with a 3 cm defect showed of 50% bone filling up the defect area. In the group of 4.5 cm defect of skeletal immature sheep, some bone formation was seen in 5/20 animals. While no bone formation was seen in the 4.5 cm defect in skeletal mature animals. Combined these data confirms that not only the size of the defect, but also the age of the animal and the surrounding environment play a crucial role in the selection of a suitable animal model for preclinical testing.

A more straight forward sheep model of critical sized middiaphyseal femur defects was used to evaluate an engineered periosteal substitute, intended for the treatment of a critical sized long bone defect after tumor resection (Knothe Tate et al. 2011). The periosteal substitute consisted of an isotropic elastomer alone, the isotropic elastomer with collagen membranes, the isotropic elastomer with a collagen membrane and autologous periosteal cells or the elastomer with autologous periosteal strips. Upon the creation of 2.54 cm segmental defects in the middiaphyseal femur, the 3.5 cm long periosteal substitutes designed to overlap 0.5 cm distal and proximal to the defect were immediately implanted with a 5 mm overlap with the native periosteum. Bone formation was evaluated by micro-CT and histomorphometry after 3 and 16 weeks post implantation. Improved fracture regeneration was seen in the groups with membranes incorporating periosteal factors (cells or strips) as compared to isotropic control membranes made of the same material or membranes with only collagen strips. Quantification based on histological evaluation further confirmed an improved bone regeneration in membranes containing the full periosteal strips in comparison to the periosteal cells. Consequently, these data suggests that not only the periosteal progenitor cells, but also the periosteal niche as such is important for the regenerative potential of the periosteum. These findings are in line with recent literature where the periosteal homing in mice was studied and specifically the matrix protein periostin was identified as crucial for the regenerative potential of periosteal cells (Duchamp de Lageneste et al. 2018).

12 Clinical Evaluation of Periosteal Cells

The quest to identify and characterize the different progenitor cell populations present in the periosteum may just have started, but their unique capabilities have already brought them to the clinical setting. Upon conducting a search for clinical trials containing the keywords periosteum/periosteal, seven trials can be found. Five of these are already completed, one is recruiting and a final study is yet to be started (Clinicaltrials.gov 2019). In a completed study from 2018, the aim was to evaluate the possible benefit on wound healing and flap stability of a periosteum inclusion, comparing a “split-full-split” thickness flap elevation versus a “split” thickness approach for the treatment of isolated-type gingival recessions in the upper jaw. In another completed study from 2007, the use of periosteum versus bone fixation was

evaluated in forehead lift. In this procedure, variable methods of fixation have been described with different rates of success. In a third completed study from 2008, an arthroscopic-assisted posterior cruciate ligament (PCL) reconstruction was performed using a femoral knot/press-fit technique with a periosteum-enveloping graft in a tibial tunnel. The investigators evaluated the results of patients with PCL ruptures, who underwent PCL reconstruction using this unique technique. In a study from 2015, a study directed towards the evaluation of two autogenous regenerative materials, marginal periosteal pedicle graft (MPP) and platelet rich fibrin (PRF) as membrane barriers for treating intrabony defects was evaluated. This study was conducted due to the periosteum's reported significant clinical outcomes, but the limited availability of the periosteum makes it necessary to evaluate other autogenous alternatives such as PRF that could offer predictable outcomes. In another study completed 2014, clinical and radiographic evaluation of autogenous periosteal pedicle graft in comparison with collagen membrane for management of periodontal intrabony defects was evaluated. Encouragingly, the periosteal group showed reduced defects at 6 months *post* treatment. Unfortunately, apart from this study, no results are posted in any of the other completed studies. In another currently recruiting study, periosteum in the recession defect site for gingival recession will be used as an autologous graft after raising a flap and the results will be compared with a group treated by the current standard treatment, coronally advanced flap with subepithelial connective tissue graft. In the final study that is a prospective interventional study yet to start recruitment, the effectiveness of a periosteal pedicle will be evaluated and compared as grafting technique in combination with an egg shell derived nano hydroxyapatite as regenerative graft material for regeneration of intrabony defects. In a currently active study, evaluation of the clinical efficacy and safety of autologous osteo-periosteal cylinder graft transplantation for Hepple V osteochondral lesions of the talus is done. Half of participants will receive autologous osteo-periosteal cylinder graft transplantation, while the other will receive osteochondral graft transplantation as a control group.

In conclusion, the completed, currently ongoing or planned studies using periosteum covers a broad range of conditions as well as treatments. Even though the first study was completed in 2008, it was only in recent years that the number of studies has started to increase. This may be linked to the increasing general knowledge of the periosteum and the potency of the progenitor cells within. However, it is surprising that there are currently no studies registered for the treatment of critical size bone fractures, or periosteum derived cell-based implants for critical bone defects. Since commonly, all registered studies used the periosteum as a tissue, rather than the isolated and expanded cells. When searching PubMed in December 2019 with the keyword "periosteum" 8798 articles are found, while 10,003 articles show up with "periosteal" as a keyword. In addition, when searching for "periosteal cells" 2022 articles are found, while 533 are found on the search of "periosteum derived cells." These search results suggest that the use of periosteum derived cells is still limited and mainly restricted to research laboratories. However, with the increasing knowledge regarding their unique potential, their role in tissue engineering strategies will most likely increase.

13 The Potential of Periosteum Derived Cells as In vitro Models to Evaluate Treatments for Specific Patients

The field of cancer biology is in general a great inspiration for regenerative medicine. In cancer, a small population of cells self-renew to replenish the growing cancer. In order to eliminate the disease, these proliferating cells need to be eliminated. To achieve this, it requires that the specific mother cell is identified, the proliferative mechanism needs to be understood and a treatment that stops the proliferation of the specific cell, in the explicit setting, could be developed for an effective treatment. For this, patient specific models have been created in order to define personalized treatment (Aleman and Skardal 2019). A similar personalized medicine approach was used to define a treatment alternative to rescue Fibroblast Growth Factor Receptor (FGFR)3 skeletal dysplasia phenotypes through the generation of patient-specific induced pluripotent stem cells (iPSCs) which functioned as *in vitro* models in the identification of a suitable treatment (Yamashita et al. 2014). Even though iPSCs represent a great *in vitro* model since their (epi)-genetic memory remains, the generation of iPSCs requires a long culture period, the process is not very efficient and costly, and safety issues remain. Combined, these factors limit their use as a cell-population for a regenerative implant. However, *in vitro* models could be very interesting when the goal is to target the intrinsic repair mechanisms by the recruitment of endogenous stem/progenitor populations into damaged tissue followed by cell proliferation, differentiation and tissue metabolic activity (Luyten and Roberts 2018). By understanding how the patients cells respond to specific treatments *in vitro*, the *in vivo* outcome can me optimized with increased potential for success (Aleman and Skardal 2019). For this, a small sample of their periosteum would be sufficient to optimize a suitable treatment based on the status of their periosteal cells, their cell surface markers, and potential mutations that can be characterized *in vitro, prior to in situ* intervention.

14 Conclusions

Regenerative constructs that mimic our body's natural regenerative system represent an attractive strategy for restoring the function of damaged organs or tissues where currently no reliable treatment is available (Ho-Shui-Ling et al. 2018; Langer and Vacanti 1993; Ma et al. 2014). Key in the construct's success lies in a bioinspired design, typically using appropriate and potent progenitor cells, which upon proliferation and differentiation into tissue intermediates direct tissue repair *in vivo* together with the available host environment to facilitate integration (Ingber et al. 2006; Lenas et al. 2009a). It is clear that periosteum derived progenitor cells play a crucial role in bone development, maintenance, and fracture healing. Recent progress made in the field of periosteal progenitor cell identification has begun to unravel the specific populations available, and with this information, treatments to target these to direct *in vivo* tissue formation have only just begun. Based on this,

periosteum derived cells represent a very attractive cell source in the development of *in situ* treatment strategies or cell-based constructs for (large) skeletal defects.

References

Agata H, Asahina I, Yamazaki Y, Uchida M, Shinohara Y, Honda MJ, Kagami H, Ueda M (2007) Effective bone engineering with periosteum-derived cells. J Dent Res 86:79–83

Aleman J, Skardal A (2019) A multi-site metastasis-on-a-chip microphysiological system for assessing metastatic preference of cancer cells. Biotechnol Bioeng 116:936–944

Allen MR, Hock JM, Burr DB (2004) Periosteum: biology, regulation, and response to osteoporosis therapies. Bone 35:1003–1012

Almeida CR, Caires HR, Vasconcelos DP, Barbosa MA (2016) NAP-2 secreted by human NK cells can stimulate mesenchymal stem/stromal cell recruitment. Stem Cell Rep 6:466–473

Ambrosi TH, Longaker MT, Chan CKF (2019) A revised perspective of skeletal stem cell biology. Front Cell Dev Biol 7:189

Archer CW, Dowthwaite GP, Francis-West P (2003) Development of synovial joints. Birth Defects Res C Embryo Today 69:144–155

Astori G, Amati E, Bambi F, Bernardi M, Chieregato K, Schafer R, Sella S, Rodeghiero F (2016) Platelet lysate as a substitute for animal serum for the ex-vivo expansion of mesenchymal stem/stromal cells: present and future. Stem Cell Res Ther 7:93

Baker M (2016) Reproducibility: respect your cells! Nature 537:433–435

Bakker AD, Schrooten J, van Cleynenbreugel T, Vanlauwe J, Luyten J, Schepers E, Dubruel P, Schacht E, Lammens J, Luyten FP (2008) Quantitative screening of engineered implants in a long bone defect model in rabbits. Tissue Eng Part C Methods 14:251–260

Barak MM, Lieberman DE, Hublin JJ (2013) Of mice, rats and men: trabecular bone architecture in mammals scales to body mass with negative allometry. J Struct Biol 183:123–131

Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, Kokkaliaris KD, Mercier F, Tabaka M, Hofree M et al (2019) A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. Cell 177:1915–1932 e1916

Ben Azouna N, Jenhani F, Regaya Z, Berraeis L, Ben Othman T, Ducrocq E, Domenech J (2012) Phenotypical and functional characteristics of mesenchymal stem cells from bone marrow: comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum. Stem Cell Res Ther 3:6

Berninger MT, Wexel G, Rummeny EJ, Imhoff AB, Anton M, Henning TD, Vogt S (2013) Treatment of osteochondral defects in the rabbit's knee joint by implantation of allogeneic mesenchymal stem cells in fibrin clots. J Vis Exp JoVE 75:e4423

Bianco P, Robey PG (2015) Skeletal stem cells. Development 142:1023–1027

Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY (2013) The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19:35–42

Bjurholm A, Kreicbergs A, Terenius L, Goldstein M, Schultzberg M (1988) Neuropeptide Y-, tyrosine hydroxylase- and vasoactive intestinal polypeptide-immunoreactive nerves in bone and surrounding tissues. J Auton Nerv Syst 25:119–125

Blaszczyk B, Kaspera W, Ficek K, Kajor M, Binkowski M, Stodolak-Zych E, Grajoszek A, Stojko J, Bursig H, Ladzinski P (2018) Effects of polylactide copolymer implants and platelet-rich plasma on bone regeneration within a large calvarial defect in sheep. Biomed Res Int 2018:4120471

Bocker W, Docheva D, Prall WC, Egea V, Pappou E, Rossmann O, Popov C, Mutschler W, Ries C, Schieker M (2008) IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells. J Mol Med 86:1183–1192

Bolander J, Ji W, Geris L, Bloemen V, Chai YC, Schrooten J, Luyten FP (2016) The combined mechanism of bone morphogenetic protein- and calcium phosphate-induced skeletal tissue formation by human periosteum derived cells. Eur Cell Mater 31:11–25

Bolander J, Ji W, Leijten J, Teixeira LM, Bloemen V, Lambrechts D, Chaklader M, Luyten FP (2017) Healing of a large Long-bone defect through serum-free in vitro priming of human periosteum-derived cells. Stem Cell Rep 8:758–772

Bolander J, Herpelinck T, Chaklader M, Gklava C, Geris L, Luyten FP (2019) Single-cell characterization and metabolic profiling of in vitro cultured human skeletal progenitors with enhanced in vivo bone forming capacity. Stem Cells Transl Med 9(3):389–402

Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H (2011) Platelets at work in primary hemostasis. Blood Rev 25:155–167

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650

Castaneda S, Largo R, Calvo E, Rodriguez-Salvanes F, Marcos ME, Diaz-Curiel M, Herrero-Beaumont G (2006) Bone mineral measurements of subchondral and trabecular bone in healthy and osteoporotic rabbits. Skelet Radiol 35:34–41

Chan CK, Seo EY, Chen JY, Lo D, McArdle A, Sinha R, Tevlin R, Seita J, Vincent-Tompkins J, Wearda T et al (2015) Identification and specification of the mouse skeletal stem cell. Cell 160:285–298

Chan CKF, Gulati GS, Sinha R, Tompkins JV, Lopez M, Carter AC, Ransom RC, Reinisch A, Wearda T, Murphy M et al (2018) Identification of the human skeletal stem cell. Cell 175:43–56 e21

Clinicaltrials.gov (2019) Periosteum in clinical trials. Access date 16 Dec 2019. https://clinicaltrials.gov/ct2/results?cond=periosteum&term=&cntry=&state=&city=&dist=

Colnot C (2009) Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. J Bone Miner Res Off J Am Soc Bone Miner Res 24:274–282

Cooper GM, Mooney MP, Gosain AK, Campbell PG, Losee JE, Huard J (2010) Testing the critical size in calvarial bone defects: revisiting the concept of a critical-size defect. Plast Reconstr Surg 125:1685–1692

Croitoru-Lamoury J, Lamoury FM, Caristo M, Suzuki K, Walker D, Takikawa O, Taylor R, Brew BJ (2011) Interferon-gamma regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (IDO). PLoS One 6:e14698

Davies DV (1963) The anatomy and physiology of joints. Physiotherapy 49:3–7

Debnath S, Yallowitz AR, McCormick J, Lalani S, Zhang T, Xu R, Li N, Liu Y, Yang YS, Eiseman M et al (2018) Discovery of a periosteal stem cell mediating intramembranous bone formation. Nature 562:133–139

Diaz-Flores L, Gutierrez R, Lopez-Alonso A, Gonzalez R, Varela H (1992) Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. Clin Orthop Relat Res 275:280–286

Dorronsoro A, Ferrin I, Salcedo JM, Jakobsson E, Fernandez-Rueda J, Lang V, Sepulveda P, Fechter K, Pennington D, Trigueros C (2014) Human mesenchymal stromal cells modulate T-cell responses through TNF-alpha-mediated activation of NF-kappaB. Eur J Immunol 44:480–488

Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, Lataillade JJ (2005) Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. J Cell Physiol 205:228–236

Duchamp de Lageneste O, Julien A, Abou-Khalil R, Frangi G, Carvalho C, Cagnard N, Cordier C, Conway SJ, Colnot C (2018) Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. Nat Commun 9:773

Dwek JR (2010) The periosteum: what is it, where is it, and what mimics it in its absence? Skelet Radiol 39:319–323

Einhorn TA (1998) The cell and molecular biology of fracture healing. Clin Orthop Relat Res (355 Suppl):S7–S21. https://doi.org/10.1097/00003086-199810001-00003

Eyckmans J, Roberts SJ, Schrooten J, Luyten FP (2010) A clinically relevant model of osteoinduction: a process requiring calcium phosphate and BMP/Wnt signalling. J Cell Mol Med 14:1845–1856

Eyckmans J, Roberts SJ, Bolander J, Schrooten J, Chen CS, Luyten FP (2013) Mapping calcium phosphate activated gene networks as a strategy for targeted osteoinduction of human progenitors. Biomaterials 34:4612–4621

Fazzina R, Iudicone P, Fioravanti D, Bonanno G, Totta P, Zizzari IG, Pierelli L (2016) Potency testing of mesenchymal stromal cell growth expanded in human platelet lysate from different human tissues. Stem Cell Res Ther 7:122

Fitzsimmons JS, Sanyal A, Gonzalez C, Fukumoto T, Clemens VR, O'Driscoll SW, Reinholz GG (2004) Serum-free media for periosteal chondrogenesis in vitro. J Orthop Res 22: 716–725

Gupta P, Hall GN, Geris L, Luyten FP, Papantoniou I (2019) Human platelet lysate improves bone forming potential of human progenitor cells expanded in microcarrier-based dynamic culture. Stem Cells Transl Med 8:810–821

Hayashi O, Katsube Y, Hirose M, Ohgushi H, Ito H (2008) Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. Calcif Tissue Int 82:238–247

He A, Liu L, Luo X, Liu Y, Liu Y, Liu F, Wang X, Zhang Z, Zhang W, Liu W et al (2017a) Repair of osteochondral defects with in vitro engineered cartilage based on autologous bone marrow stromal cells in a swine model. Sci Rep 7:40489

He X, Bougioukli S, Ortega B, Arevalo E, Lieberman JR, McMahon AP (2017b) Sox9 positive periosteal cells in fracture repair of the adult mammalian long bone. Bone 103:12–19

Heathman TR, Nienow AW, McCall MJ, Coopman K, Kara B, Hewitt CJ (2015) The translation of cell-based therapies: clinical landscape and manufacturing challenges. Regen Med 10:49–64

Horner EA, Kirkham J, Wood D, Curran S, Smith M, Thomson B, Yang XB (2010) Long bone defect models for tissue engineering applications: criteria for choice. Tissue Eng Part B Rev 16:263–271

Ho-Shui-Ling A, Bolander J, Rustom LE, Johnson AW, Luyten FP, Picart C (2018) Bone regeneration strategies: engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives. Biomaterials 180:143–162

Ingber DE, Mow VC, Butler D, Niklason L, Huard J, Mao J, Yannas I, Kaplan D, Vunjak-Novakovic G (2006) Tissue engineering and developmental biology: going biomimetic. Tissue Eng 12:3265–3283

International Stem Cell Initiative C, Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, Christie M, Ford A, Fox V, Gokhale PJ et al (2010) Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. In Vitro Cell Dev Biol Anim 46:247–258

Ionescu A, Kozhemyakina E, Nicolae C, Kaestner KH, Olsen BR, Lassar AB (2012) FoxA family members are crucial regulators of the hypertrophic chondrocyte differentiation program. Dev Cell 22:927–939

Isern J, Garcia-Garcia A, Martin AM, Arranz L, Martin-Perez D, Torroja C, Sanchez-Cabo F, Mendez-Ferrer S (2014) The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. elife 3:e03696

Ishikawa M, Ito H, Kitaori T, Murata K, Shibuya H, Furu M, Yoshitomi H, Fujii T, Yamamoto K, Matsuda S (2014) MCP/CCR2 signaling is essential for recruitment of mesenchymal progenitor cells during the early phase of fracture healing. PLoS One 9:e104954

Ito Y, Ochi M, Adachi N, Sugawara K, Yanada S, Ikada Y, Ronakorn P (2005) Repair of osteochondral defect with tissue-engineered chondral plug in a rabbit model. Arthroscopy 21:1155–1163

Ji W, Wang H, van den Beucken JJ, Yang F, Walboomers XF, Leeuwenburgh S, Jansen JA (2012) Local delivery of small and large biomolecules in craniomaxillofacial bone. Adv Drug Deliv Rev 64:1152–1164

Jonsdottir-Buch SM, Lieder R, Sigurjonsson OE (2013) Platelet lysates produced from expired platelet concentrates support growth and osteogenic differentiation of mesenchymal stem cells. PLoS One 8:e68984

Jung S, Panchalingam KM, Rosenberg L, Behie LA (2012) Ex vivo expansion of human mesenchymal stem cells in defined serum-free media. Stem Cells Int 2012:123030

Kitaori T, Ito H, Schwarz EM, Tsutsumi R, Yoshitomi H, Oishi S, Nakano M, Fujii N, Nagasawa T, Nakamura T (2009) Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. Arthritis Rheum 60:813–823

Knothe Tate ML, Chang H, Moore SR, Knothe UR (2011) Surgical membranes as directional delivery devices to generate tissue: testing in an ovine critical sized defect model. PLoS One 6: e28702

Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S et al (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309

Kudva AK, Luyten FP, Patterson J (2018) RGD-functionalized polyethylene glycol hydrogels support proliferation and in vitro chondrogenesis of human periosteum-derived cells. J Biomed Mater Res A 106:33–42

Kudva AK, Dikina AD, Luyten FP, Alsberg E, Patterson J (2019) Gelatin microspheres releasing transforming growth factor drive in vitro chondrogenesis of human periosteum derived cells in micromass culture. Acta Biomater 90:287–299

Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, Mizoguchi T, Wei Q, Lucas D, Ito K et al (2013) Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 502:637–643

Lambrechts T, Papantoniou I, Rice B, Schrooten J, Luyten FP, Aerts JM (2016) Large-scale progenitor cell expansion for multiple donors in a monitored hollow fibre bioreactor. Cytotherapy 18:1219–1233

Lammens J, Bauduin G, Driesen R, Moens P, Stuyck J, De Smet L, Fabry G (1998) Treatment of nonunion of the humerus using the Ilizarov external fixator. Clin Orthop Relat Res 353:223–230

Lammens J, Marechal M, Geris L, Van der Aa J, Van Hauwermeiren H, Luyten FP, Delport H (2017) Warning about the use of critical-size defects for the translational study of bone repair: analysis of a sheep Tibial model. Tissue Eng Part C Methods 23:694–699

Langer R, Vacanti JP (1993) Tissue engineering. Science 260:920–926

Lehmann W, Edgar CM, Wang K, Cho TJ, Barnes GL, Kakar S, Graves DT, Rueger JM, Gerstenfeld LC, Einhorn TA (2005) Tumor necrosis factor alpha (TNF-alpha) coordinately regulates the expression of specific matrix metalloproteinases (MMPS) and angiogenic factors during fracture healing. Bone 36:300–310

Lenas P, Moos M, Luyten FP (2009a) Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part I: from three-dimensional cell growth to biomimetics of in vivo development. Tissue Eng Part B Rev 15:381–394

Lenas P, Moos M, Luyten FP (2009b) Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part II: from genes to networks: tissue engineering from the viewpoint of systems biology and network science. Tissue Eng Part B Rev 15:395–422

Li W, Kohara H, Uchida Y, James JM, Soneji K, Cronshaw DG, Zou YR, Nagasawa T, Mukouyama YS (2013) Peripheral nerve-derived CXCL12 and VEGF-A regulate the patterning of arterial vessel branching in developing limb skin. Dev Cell 24:359–371

Li Z, Meyers CA, Chang L, Lee S, Li Z, Tomlinson R, Hoke A, Clemens TL, James AW (2019) Fracture repair requires TrkA signaling by skeletal sensory nerves. J Clin Invest 129:5137–5150

Luyten FP, Roberts SJ (2018) Close to the bone – in search of the skeletal stem cell. Nat Rev Rheumatol 14:687–688

Ma J, Both SK, Yang F, Cui FZ, Pan J, Meijer GJ, Jansen JA, van den Beucken JJ (2014) Concise review: cell-based strategies in bone tissue engineering and regenerative medicine. Stem Cells Transl Med 3:98–107

Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, Carmeliet G, Kronenberg HM (2010) Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev Cell 19:329–344

Manabe N, Kawaguchi H, Chikuda H, Miyaura C, Inada M, Nagai R, Nabeshima Y, Nakamura K, Sinclair AM, Scheuermann RH et al (2001) Connection between B lymphocyte and osteoclast differentiation pathways. J Immunol 167:2625–2631

Mapara M, Thomas BS, Bhat KM (2012) Rabbit as an animal model for experimental research. Dent Res J 9:111–118

Marecic O, Tevlin R, McArdle A, Seo EY, Wearda T, Duldulao C, Walmsley GG, Nguyen A, Weissman IL, Chan CK et al (2015) Identification and characterization of an injury-induced skeletal progenitor. Proc Natl Acad Sci U S A 112:9920–9925

Masquelet AC, Begue T (2010) The concept of induced membrane for reconstruction of long bone defects. Orthop Clin North Am 41:27–37; table of contents

Matthyssen S, Ni Dhubhghaill S, Van Gerwen V, Zakaria N (2017) Xeno-free cultivation of mesenchymal stem cells from the corneal Stroma. Invest Ophthalmol Vis Sci 58:2659–2665

McKibbin B (1978) The biology of fracture healing in long bones. J Bone Joint Surg (Br) 60-B:150–162

Mendes LF, Katagiri H, Tam WL, Chai YC, Geris L, Roberts SJ, Luyten FP (2018) Advancing osteochondral tissue engineering: bone morphogenetic protein, transforming growth factor, and fibroblast growth factor signaling drive ordered differentiation of periosteal cells resulting in stable cartilage and bone formation in vivo. Stem Cell Res Ther 9:42

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

Mendez-Ferrer S, Scadden DT, Sanchez-Aguilera A (2015) Bone marrow stem cells: current and emerging concepts. Ann N Y Acad Sci 1335:32–44

Mizuhashi K, Ono W, Matsushita Y, Sakagami N, Takahashi A, Saunders TL, Nagasawa T, Kronenberg HM, Ono N (2018) Resting zone of the growth plate houses a unique class of skeletal stem cells. Nature 563:254–258

Mizuno K, Mineo K, Tachibana T, Sumi M, Matsubara T, Hirohata K (1990) The osteogenetic potential of fracture haematoma. Subperiosteal and intramuscular transplantation of the haematoma. J Bone Joint Surg (Br) 72:822–829

Moore SR, Milz S, Knothe Tate ML (2014) Periosteal thickness and cellularity in mid-diaphyseal cross-sections from human femora and tibiae of aged donors. J Anat 224:142–149

Moreira CA, Dempster DW, Baron R (2000) Anatomy and ultrastructure of bone - Histogenesis, growth and remodeling. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, Dungan K, Grossman A, Hershman JM, Kaltsas G, Koch C, Kopp P et al (eds) Endotext [Internet]. South Dartmouth (MA)

Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, Hiratsu E, Suzuki S, Miyauchi-Hara C, Nagoshi N, Sunabori T et al (2009) Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J Exp Med 206:2483–2496

Newton PT, Li L, Zhou B, Schweingruber C, Hovorakova M, Xie M, Sun X, Sandhow L, Artemov AV, Ivashkin E et al (2019) A radical switch in clonality reveals a stem cell niche in the epiphyseal growth plate. Nature 567:234–238

Nilsson Hall G, Mendes LF, Gklava C, Geris L, Luyten FP, Papantoniou I (2020) Developmentally engineered callus organoid bioassemblies exhibit predictive in vivo Long bone healing. Adv Sci n/a:1902295

Ono N, Ono W, Mizoguchi T, Nagasawa T, Frenette PS, Kronenberg HM (2014) Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. Dev Cell 29:330–339

Ortinau LC, Wang H, Lei K, Deveza L, Jeong Y, Hara Y, Grafe I, Rosenfeld SB, Lee D, Lee B et al (2019) Identification of functionally distinct Mx1+alphaSMA+ periosteal skeletal stem cells. Cell Stem Cell 25:784–796 e785

Owen M, Friedenstein AJ (1988) Stromal stem cells: marrow-derived osteogenic precursors. CIBA Found Symp 136:42–60

Pineault KM, Song JY, Kozloff KM, Lucas D, Wellik DM (2019) Hox11 expressing regional skeletal stem cells are progenitors for osteoblasts, chondrocytes and adipocytes throughout life. Nat Commun 10:3168

Pobloth AM, Duda GN, Giesecke MT, Dienelt A, Schwabe P (2015) High-dose recombinant human bone morphogenetic protein-2 impacts histological and biomechanical properties of a cervical spine fusion segment: results from a sheep model. J Tissue Eng Regen Med 11(5):1514–1523

Reinisch A, Bartmann C, Rohde E, Schallmoser K, Bjelic-Radisic V, Lanzer G, Linkesch W, Strunk D (2007) Humanized system to propagate cord blood-derived multipotent mesenchymal stromal cells for clinical application. Regen Med 2:371–382

Rhinelander FW, Baragry R (1962) Microangiography in bone healing. I. Undisplaced closed fractures. J Bone Joint Surg Am 44-A:1273–1298

Roberts SJ, Geris L, Kerckhofs G, Desmet E, Schrooten J, Luyten FP (2011) The combined bone forming capacity of human periosteal derived cells and calcium phosphates. Biomaterials 32:4393–4405

Robey P (2017) "Mesenchymal stem cells": fact or fiction, and implications in their therapeutic use. F1000Res 6:F1000 Faculty Rev-524

Ryan JM (1979) Effect of different fetal bovine serum concentrations on the replicative life span of cultured chick cells. In Vitro 15:895–899

Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M et al (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131:324–336

Sadtler K, Wolf MT, Ganguly S, Moad CA, Chung L, Majumdar S, Housseau F, Pardoll DM, Elisseeff JH (2019) Divergent immune responses to synthetic and biological scaffolds. Biomaterials 192:405–415

Salazar VS, Gamer LW, Rosen V (2016) BMP signalling in skeletal development, disease and repair. Nat Rev Endocrinol 12:203–221

Salazar VS, Capelo LP, Cantu C, Zimmerli D, Gosalia N, Pregizer S, Cox K, Ohte S, Feigenson M, Gamer L et al (2019) Reactivation of a developmental Bmp2 signaling center is required for therapeutic control of the murine periosteal niche. elife 8:e42386

Salvade A, Della Mina P, Gaddi D, Gatto F, Villa A, Bigoni M, Perseghin P, Serafini M, Zatti G, Biondi A et al (2010) Characterization of platelet lysate cultured mesenchymal stromal cells and their potential use in tissue-engineered osteogenic devices for the treatment of bone defects. Tissue Eng Part C Methods 16:201–214

Schell H, Duda GN, Peters A, Tsitsilonis S, Johnson KA, Schmidt-Bleek K (2017) The haematoma and its role in bone healing. J Exp Orthop 4:5

Schinhan M, Gruber M, Vavken P, Dorotka R, Samouh L, Chiari C, Gruebl-Barabas R, Nehrer S (2012) Critical-size defect induces unicompartmental osteoarthritis in a stable ovine knee. J Orthop Res 30:214–220

Segal AW (2005) How neutrophils kill microbes. Annu Rev Immunol 23:197–223

Shahdadfar A, Fronsdal K, Haug T, Reinholt FP, Brinchmann JE (2005) In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells 23:1357–1366

Shi Y, He G, Lee WC, McKenzie JA, Silva MJ, Long F (2017) Gli1 identifies osteogenic progenitors for bone formation and fracture repair. Nat Commun 8:2043

Shields LB, Raque GH, Glassman SD, Campbell M, Vitaz T, Harpring J, Shields CB (2006) Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion. Spine 31:542–547

Song MK, Lee ZH, Kim HH (2015) Adseverin mediates RANKL-induced osteoclastogenesis by regulating NFATc1. Exp Mol Med 47:e199

Stockmann P, Park J, von Wilmowsky C, Nkenke E, Felszeghy E, Dehner JF, Schmitt C, Tudor C, Schlegel KA (2012) Guided bone regeneration in pig calvarial bone defects using autologous mesenchymal stem/progenitor cells – a comparison of different tissue sources. J Craniomaxillofac Surg 40:310–320

Struillou X, Boutigny H, Soueidan A, Layrolle P (2010) Experimental animal models in periodontology: a review. Open Dent J 4:37–47

Strunk D, Lozano M, Marks DC, Loh YS, Gstraunthaler G, Schennach H, Rohde E, Laner-Plamberger S, Oller M, Nystedt J et al (2018) International forum on GMP-grade human platelet lysate for cell propagation: summary. Vox Sang 113:80–87

Stuart RC, Hehir D (1992) Comparative efficacy of cimetidine, famotidine, ranitidine, and mylanta in postoperative stress ulcers. Gastroenterology 102:1091

Szpalski C, Barr J, Wetterau M, Saadeh PB, Warren SM (2010) Cranial bone defects: current and future strategies. Neurosurg Focus 29:E8

Tavassoli M, Crosby WH (1968) Transplantation of marrow to extramedullary sites. Science 161:54–56

Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V (2006) BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 38:1424–1429

van Gastel N, Torrekens S, Roberts SJ, Moermans K, Schrooten J, Carmeliet P, Luttun A, Luyten FP, Carmeliet G (2012) Engineering vascularized bone: osteogenic and proangiogenic potential of murine periosteal cells. Stem Cells 30:2460–2471

van Griensven M (2015) Preclinical testing of drug delivery systems to bone. Adv Drug Deliv Rev

Vashishth D (2008) Small animal bone biomechanics. Bone 43:794–797

Verbeeck L, Geris L, Tylzanowski P, Luyten FP (2019) Uncoupling of in-vitro identity of embryonic limb derived skeletal progenitors and their in-vivo bone forming potential. Sci Rep 9:5782

Via AG, Frizziero A, Oliva F (2012) Biological properties of mesenchymal stem cells from different sources. Muscle Ligaments Tendons J 2:154–162

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. Osteoarthr Cartil 10:199–206

Wang X, Mabrey JD, Agrawal CM (1998) An interspecies comparison of bone fracture properties. Biomed Mater Eng 8:1–9

Wehrhan F, Amann K, Molenberg A, Lutz R, Neukam FW, Schlegel KA (2013) Critical size defect regeneration using PEG-mediated BMP-2 gene delivery and the use of cell occlusive barrier membranes - the osteopromotive principle revisited. Clin Oral Implants Res 24:910–920

Worthley DL, Churchill M, Compton JT, Tailor Y, Rao M, Si Y, Levin D, Schwartz MG, Uygur A, Hayakawa Y et al (2015) Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. Cell 160:269–284

Xian CJ, Zhou FH, McCarty RC, Foster BK (2004) Intramembranous ossification mechanism for bone bridge formation at the growth plate cartilage injury site. J Orthop Res 22:417–426

Xu S, Lin K, Wang Z, Chang J, Wang L, Lu J, Ning C (2008) Reconstruction of calvarial defect of rabbits using porous calcium silicate bioactive ceramics. Biomaterials 29:2588–2596

Yamashita A, Morioka M, Kishi H, Kimura T, Yahara Y, Okada M, Fujita K, Sawai H, Ikegawa S, Tsumaki N (2014) Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature 513:507–511

Yamashita A, Morioka M, Yahara Y, Okada M, Kobayashi T, Kuriyama S, Matsuda S, Tsumaki N (2015) Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs. Stem Cell Rep 4:404–418

Ye L, Mishina Y, Chen D, Huang H, Dallas SL, Dallas MR, Sivakumar P, Kunieda T, Tsutsui TW, Boskey A et al (2005) Dmp1-deficient mice display severe defects in cartilage formation responsible for a chondrodysplasia-like phenotype. J Biol Chem 280:6197–6203

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 327:449–462

Yu N, Plachokova A, Yang F, Walboomers X, Jansen J (2013) Engineering of dental tissues: scaffolds and preclinical models. In: Stem cells in craniofacial development, regeneration and repair. Wiley-Blackwell Press

Zhou BO, Yue R, Murphy MM, Peyer JG, Morrison SJ (2014) Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. Cell Stem Cell 15:154–168

Bone-Marrow-Derived Mesenchymal Stromal Cells: From Basic Biology to Applications in Bone Tissue Engineering and Bone Regeneration

Janja Zupan, Daniel Tang, Richard O. C. Oreffo, Heinz Redl, and Darja Marolt Presen

Contents

J. Zupan
Department of Clinical Biochemistry, Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia
e-mail: Janja.Zupan@ffa.uni-lj.si

D. Tang · R. O. C. Oreffo
Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton, UK
e-mail: dkot1e14@southamptonalumni.ac.uk; Richard.Oreffo@soton.ac.uk

H. Redl · D. Marolt Presen (✉)
AUVA Trauma Research Center, Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria
e-mail: Heinz.Redl@trauma.lbg.ac.at; Darja.Marolt@traumal.lbg.ac.at

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_7

Abstract

Bone marrow provides a rich source of mesenchymal stromal cells (MSCs), which have the remarkable capacity for cell and tissue regeneration. Since their initial discovery in the guinea pig almost 50 years ago, bone-marrow-derived MSCs have been extensively studied in animals and humans. Several subpopulations have been characterized with the aim to isolate, enrich, and identify the cells with stem-cell properties and immunomodulatory actions, which are important for regenerative medicine. In this chapter, we review the properties of bone-marrow-derived MSCs with a focus on the preclinical setting and discuss their applications for bone tissue engineering and bone regeneration.

1 Introduction: Definition and History of Bone-Marrow-Derived Mesenchymal Stromal Cells

Bone marrow is the most studied tissue source of mesenchymal stromal cells (MSCs), which are also typically referred to as mesenchymal "stem" cells. Since their identification in the bone marrow of the guinea pig (Friedenstein et al. 1970), MSCs derived from bone marrow of animals and humans have been studied extensively across a variety of biomedical disciplines (Čamernik et al. 2018; Marolt Presen et al. 2019). Initially, bone-marrow stromal cells were defined by Friedenstein and colleagues as colony-forming and osteogenic fibroblast-like bone-marrow stromal cells (Friedenstein et al. 1970).

The term "mesenchymal stem cells" was coined by Arnold Caplan some 20 years later, who defined them as pluripotent progenitor clonogenic cells, the progeny of which can give rise to skeletal tissues such as bone, cartilage, tendons, ligaments, marrow stroma, fat, dermis, muscle, and connective tissue (Caplan 1991). Although MSCs can theoretically differentiate into a variety of end-stage skeletal tissue phenotypes, their transit from one stage to the next depends not just on their own regulatory signals (i.e., autocrine regulation), but also on local cues from the surrounding cells (i.e., paracrine regulation). Caplan indicated that the sum of these various intrinsic and extrinsic signals that define the developmental path of these cells is difficult to reconstruct in a cell-culture dish (Caplan 1991). Furthermore, Caplan envisaged that autologous MSCs, in particular, would govern the rapid and specific repair of skeletal tissues, as required in bone disorders such as bone tumors and osteoporosis (Caplan 1991). In 1999, Pittenger and colleagues demonstrated experimentally that the populations isolated from human bone marrow were

multipotent "stem" cells, that is, capable of differentiating into lineages that include cartilage, fat, tendons, muscle, and marrow stroma (Pittenger et al. 1999). However, MSC multipotency and self-renewing potential were not demonstrated robustly at this stage using in vivo assays.

Given the ease of MSC isolation, together with their potential for mitotic expansion and site-directed delivery, numerous in vitro and in vivo studies followed (e.g., Grcevic et al. 2012; Čamernik et al. 2018; Marolt Presen et al. 2019). However, there was no consensus on the methods to be used for isolation, expansion, and characterization of these cells.

Then in 2006, the International Society for Cellular Therapy (ISCT) issued a consensus position statement that proposed the minimal criteria for definition of human multipotent stromal cells from bone marrow as "MSCs" (Dominici et al. 2006): (i) they must be plastic-adherent when maintained under standard culture conditions; (ii) they must express CD73, CD90, and CD105, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules; and (iii) they must differentiate into osteoblasts, adipocytes, and chondroblasts in vitro. Even though relatively simple and straight-forward in terms of the in vitro conditions, these three criteria also encompass stromal cells and fibroblasts that do not show stemness (i.e., self-renewal and formation of differentiated progeny in vivo). Cells with similar multipotent properties were later discovered in other adult and fetal connective tissues, among which adipose tissue was extensively studied, given its ready availability (e.g., Zuk et al. 2001; Crisan et al. 2008). It was noted that MSCs have phenotypic and functional differences that depend on their tissue origin (Gimble et al. 2007; Reinisch et al. 2015), and a consensus position statement was subsequently issued to define adipose-tissue-derived mesenchymal stromal cells (Bourin et al. 2013).

In vivo animal studies proposed different theories as to the exact nature of MSCs. Sacchetti and colleagues suggested that MSCs represent subendothelial stromal cells that reside on the sinusoidal walls (Sacchetti et al. 2007), while Crisan and colleagues suggested that MSCs are progenitor cells that reside on blood vessels, the so-called "pericytes" (Crisan et al. 2008). Both of these hypotheses provided a reasonable explanation as to why vascularized tissues are a rich source of tissue-resident multipotent progenitors. However, this pericyte hypothesis was more recently challenged by Cattaneo and colleagues (Cattaneo et al. 2018). They followed the fate of pericytes and vascular smooth muscle cells of multiple organs in vivo in aging and injury models, through which they provided new evidence to show that pericytes do not behave in the same way as stem cells (Cattaneo et al. 2018).

Sachetti and colleagues also provided data that supported the view that rather than there being a uniform class of MSCs, different mesodermal tissue derivatives include distinct classes of tissue-specific committed progenitors, with potentially different developmental origins (Sacchetti et al. 2016). They showed that $CD34^{-}/CD45^{-}/CD146^{+}$ MSCs isolated from different tissues have inherently distinct transcriptomic signatures and differentiation potentials. Furthermore, using a variety of stringent differentiation assays, they demonstrated that human bone-marrow $CD146^{+}$ cells are

inherently established to generate bone and bone-marrow stroma that support hematopoiesis and include adipocytes. However, these $CD146^{+}$ cells are not myogenic and are not spontaneously chondrogenic in vivo (Sacchetti et al. 2016).

The ISCT consensus position statement from 2006 failed to generate uniform use of the term MSCs, and different publications used it as an abbreviation for "mesenchymal stem cells" or "mesenchymal stromal cells." Therefore, in a recent publication, the ISCT urged the use of the abbreviation MSCs for "mesenchymal stromal cells," in conjunction with the indication of their tissue origin, and a robust matrix of functional assays to evaluate their properties. In contrast, the term "mesenchymal stem cells" should not be used unless "*rigorous evidence for stemness exists that can be supported by both* in vitro *and* in vivo *data*" (Viswanathan et al. 2019).

The postulation that MSCs can replace and regenerate damaged mesenchymal tissues in vivo has been challenged by a number of preclinical studies, which have shown that after their transplantation, only a limited proportion of MSCs can survive and engraft within a site of tissue damage. However, it was recognized that MSCs secrete a number of molecules with trophic and immunomodulatory properties (Le Blanc et al. 2003; Krampera et al. 2006; Gao et al. 2016; Hofer and Tuan 2016; Zhao et al. 2016). This discovery of immunosuppressive functions of MSCs raised interest in their application as a therapeutic tool to suppress inflammation and to downregulate pathogenic immune responses in graft-versus-host and autoimmune diseases (Glenn 2014).

Thus, to date, numerous mechanisms have been described by which MSCs might influence both innate and adaptive immune responses. It has been shown that MSCs can act directly on immune cells, as well as via the releasing of soluble factors into the local microenvironment, which can then affect immune cells (i.e., in a paracrine fashion) (Zachar et al. 2016). It was also suggested that suppression of the first stage of tissue injury, that is, inflammation, is a prerequisite for successful tissue regeneration (Zachar et al. 2016; Regulski 2017). Indeed, in vitro and in vivo studies have suggested that the effects of MSCs on the restoration of damaged tissues and organs include various mechanisms beyond their differentiation into the cells of the target tissue. These effects have been suggested to include immunomodulation, angiogenic activity, recruitment of stem cells and endothelial progenitors, enhancement of cell proliferation and differentiation, and antiapoptotic effects, all of which will facilitate the reparative and regenerative processes (Ponte et al. 2007; Chen et al. 2008; Hofer and Tuan 2016; Oryan et al. 2017).

In clinical use, the term MSCs is even more poorly defined (Mendicino et al. 2014), and considering also that the cell therapies that patients receive are typically referred to as "stem-cell" therapies, this is even more contentious and controversial. The term "stem cells" implies that the patients receive a direct medical benefit, as these cells have, by standard definition, the capacity to differentiate into regenerating tissue-producing cells (Caplan 2017). Caplan has recently suggested that the name MSCs be changed to "medicinal signaling cells," to reflect the findings that MSCs home in on the sites of injury or disease and secrete bioactive factors that are immunomodulatory and trophic (i.e., regenerative), hence the inference that MSCs make "medicinal" drugs in situ (Caplan 2017). Thus, as an extension here, it would

be the tissue-resident stem cells of the patient that produce the new tissue following their stimulation by the bioactive factors released from the exogenously supplied MSCs (Caplan 2017).

To date, there have been more than 350 clinical trials that involve bone-marrow-derived MSC therapies for different indications. These are most commonly related to blood and lymph conditions, cancers and other neoplasms, nervous system diseases, muscle, bone, and cartilage diseases, and wound healing (ClinicalTrials.gov database; search term: "bone-marrow mesenchymal stem cells"; November 2019).

It is important to note that the term "stem cell" can be applied to a diverse group of cells that share two characteristic properties: a capacity for prolonged or unlimited self-renewal under controlled conditions and the potential to differentiate into a variety of specialized cell types**.** Within bone specifically, the term skeletal stem cell (SSC) is used to refer specifically to the self-renewing stem cells of the bone-marrow stroma that are responsible for the regenerative capacity inherent to bone. The heterogeneous population of cultured plastic-adherent cells isolated from bone marrow that are most commonly used (if not acknowledged) by researchers in the field of bone regeneration thus typically represents bone-marrow stromal cells**.**

2 Biological Properties of Bone-Marrow-Derived Mesenchymal Stromal Cells

2.1 Mesenchymal Stromal Cells Are a Key Component of the Bone-Marrow Microenvironment

Bone marrow is an anatomical location within the trabecular bone that provides a complex microenvironment where many different cell types can interact, including hematopoietic cells, MSCs, vascular cells, and neurons (Kumar et al. 2018). Adult bone marrow is the primary site of steady-state self-renewal of hematopoietic stem cells (HSCs) and their differentiation toward their mature progeny (Wei and Frenette 2018). While HSCs and their progeny are regulated by the numerous cell types present in this complex microenvironment, it is now generally accepted that MSCs are a critical constituent of the bone-marrow HSC niche, and a major contributor of a number of known "niche factors" (Wei and Frenette 2018). A decade ago, the MSC subpopulation that was identified as an essential HSC niche component was shown to be the nestin^{+} cells (Méndez-Ferrer et al. 2010). These cells contain all of the bone-marrow-fibroblast colony-forming-unit (CFU) activities and can be propagated as nonadherent "mesenspheres" that can self-renew and expand through serial transplantations in vivo. Nestin^{+} MSCs and HSCs form a structurally unique niche in the bone marrow, which is tightly regulated by local input from the surrounding microenvironment, as well as by long-distance cues from hormones and the autonomous nervous system (Méndez-Ferrer et al. 2010). The platelet-derived growth factor receptor α (PDGFRα)$^{+}$/CD51^{+} subset of nestin^{+} cells is also enriched in major HSC maintenance genes, which supports the concept that the HSC niche activity co-segregates with MSC activity (Pinho et al. 2013).

However, other populations of bone-marrow cells have also been shown to express factors that promote HSC maintenance, such as (CXCL)12-abundant reticular (CAR) cells (Omatsu et al. 2010), neural/glial antigen 2 (NG2)-positive arteriolar cells (Kunisaki et al. 2013), and endothelium- and leptin-receptor-positive perivascular cells (Ding et al. 2012). Recently, our understanding of the bone-marrow niche expanded following a detailed transcriptional blueprint of distinct vascular, perivascular, and osteogenic lineage components (Tikhonova et al. 2019). It has been demonstrated that under stress conditions, transcriptional remodeling of the niche elements occurs. This includes adipocytic skewing of perivascular cells and the loss of the vascular-endothelium-expressed Notch ligand DLL4, which results in premature induction of the myeloid transcriptional program in HSCs (Tikhonova et al. 2019).

2.2 Studies of Bone-Marrow-Derived Mesenchymal Stromal Cells in Mouse Models

2.2.1 Identification of Mesenchymal Stromal Cell Populations in Transgenic Mouse Models

Although marrow stromal cells were first identified some 50 years ago using in vitro methods (Friedenstein et al. 1970; Pittenger et al. 1999), the low frequency and lack of specific surface-marker expression precluded the identification of their in vivo counterparts. Moreover, stem cells are thought to reside within specific anatomical structures, or "niches," where the stem cells receive signals that maintain them in an undifferentiated state until induction of self-renewal is needed. The in vivo identification of MSCs in their native bone-marrow microenvironment became possible more recently with the advances in transgenic mouse models (Fig. 1). These models enable lineage tracing, to define the origin, fate, and behavior of cells in any specific tissue or organism. Furthermore, transgenic models enable cells to be traced under both physiological and pathophysiological conditions. For instance, MSC populations can be traced to identify the tissues where these populations originate from and/or reside in postnatally. In combination with injury models, such as cartilage injury (Roelofs et al. 2017) or fracture models (Zhou et al. 2014), the specific contributions of MSCs to tissue regeneration can be assessed.

In the Cre-loxP mouse model, recombination within specific cell populations is induced based on expression of the selected promoter. The basic strategy for Cre-loxP-directed gene knockout experiments is to flank, or "flox," an essential exon of the gene of interest with two loxP sites, and then to "deliver" the Cre recombinase, which excises the intervening DNA (including the exon) from the chromosome, to thus generate a null allele in all of the cells where the Cre is active (Feil et al. 2009; Kim et al. 2018). Typically, a mouse that expresses an inducible Cre recombinase in the cell type of choice is bred with a mouse that ubiquitously expresses the Rosa26 reporter gene (Soriano 1999), with the stop codon flanked by loxP sites upstream of the reporter gene. Following promoter expression in the desired cell type, the Cre

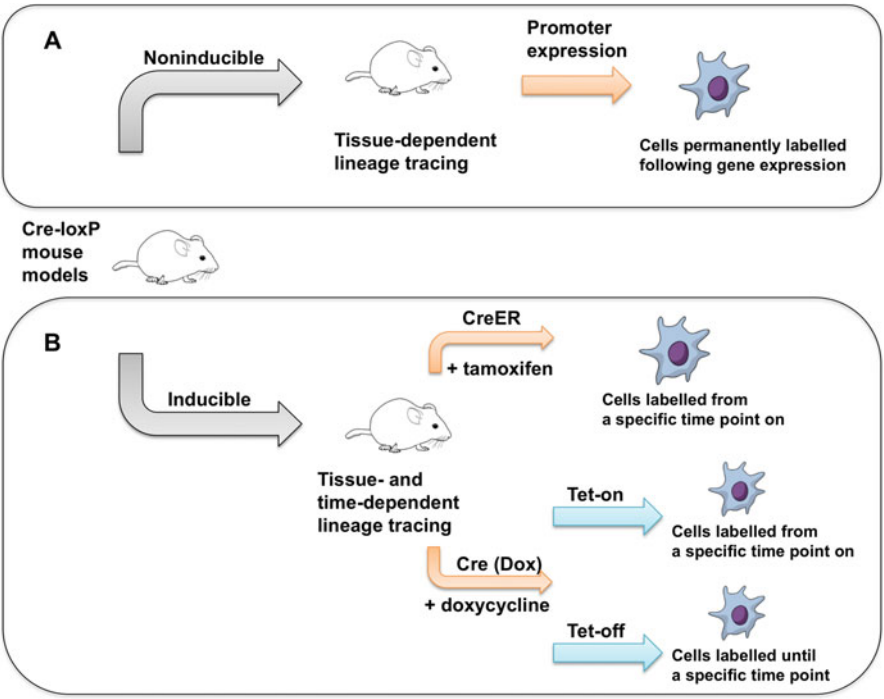


Fig. 1 Basic principles of transgenic mouse Cre-loxP systems

recombinase is expressed and excises the stop codon, which enables the Rosa26 reporter to be switched on in this particular cell type.

Cre-loxP mouse systems can be divided into two major groups, as noninducible (Fig. 1a) and inducible (Fig. 1b) systems. In noninducible systems, the specificity and timing of the recombination are controlled by expression of the selected promoter and/or enhancer. In inducible systems, the Cre recombinase can be induced by application of either tamoxifen or doxycycline. In tamoxifen-inducible systems, the estrogen receptor is fused to Cre (i.e., CreER). Administration of tamoxifen activates the estrogen receptor, which induces nuclear translocation of Cre and inactivation of the floxed gene sequence, which enables the expression of the reporter in the cells. Another temporal and cell-specific inducible system uses a tetracycline derivative, the doxycycline-inducible Cre system. This system is available as a Tet-on model, which allows doxycycline-dependent Cre gene and reporter expression, and a Tet-off model, where doxycycline results in Cre gene and reporter inactivation.

Using transgenic mouse models for MSC lineage tracing, specific populations of MSCs were identified and their roles in bone and cartilage regeneration have been determined (Table 1). Chan and colleagues elegantly mapped bone, cartilage, and bone-marrow stroma development from a population of highly pure, postnatal mouse SSCs (mSSCs) to their downstream progenitors of bone, cartilage, and

Table 1 Lineages of MSCs identified in the bone marrow using transgenic animal models

Transgenic model	Cell lineage traced	Aim of the lineage tracing	Role of the lineage cells in bone marrow	Reference
CXCL12-GFP MxCre-CXCR4$^{f/null}$	CXCL12-abundant reticular (CAR) cells	To identify the regulatory mechanism of niches for HSC maintenance	CAR cells are a key component of HSC niches in adult bone marrow	Sugiyama et al. 2006
αSMACreERT2; Cherry	Smooth muscle α-actin (αSMA) lineage cells	The role of αSMA-expressing cells in bone formation and repair	Source of fibrocartilage, skeletal progenitors in fracture-healing model	Grcevic et al. 2012
Nestin-GFP Nes-CreERT2; GFP	Nestin lineage cells	The physiological contribution of nestin-expressing MSCs to skeletal formation	Contribution to osteoblasts, osteocytes and chondrocytes during skeleton formation and remodeling	Méndez-Ferrer et al. 2010
LepR-Cre/ Tomato/Nes-GFP	Nestin and LepR lineage cells	To define the overlap between nestin and leptin receptor-expressing cells and their role in bone marrow	LepR$^+$ cells largely overlap with Nes-GFP$^+$ cells. Nestin$^+$ MSCs are an important constituent of the human fetal HSC niche	Pinho et al. 2013
Ocn-Cre-ER; YFP Mx1-Cre;YFP	Osteocalcin lineage cells; Mx1 lineage cells	To define mature osteoblasts, and their turnover in adult bone maintenance and regeneration	Mx1$^+$ cells respond to tissue stress and migrate to sites of injury, to supply new osteoblasts during fracture healing	Park et al. 2012
Osx-CreERT2; Tomato	Osterix lineage cells in fetal, perinatal and adult bone marrow	To define the developmental origin and hierarchical relationship of osteoblast progenitors defined by osterix expression	Osterix$^+$ progenitors contribute three distinct waves: in fetal bone, to nascent bone tissues and transient stromal cells; perinatally to osteolineages and long-lived stromal cells, which have characteristics of Nestin-GFP$^+$ cells;	Mizoguchi et al. 2014

(continued)

Table 1 (continued)

Transgenic model	Cell lineage traced	Aim of the lineage tracing	Role of the lineage cells in bone marrow	Reference
			and in adult bone marrow to osteolineage cells	
LepR-Cre; Tomato LepR-Cre; Tomato;Col2.3-GFP Prx1-Cre; Tomato LepR-Cre;Pten$^{fl/fl}$	LepR lineage cells in bone marrow	To identify LepR-expressing cells and their role in bone marrow	LepR^{+} cells are a major source of HSC niche factors as well as a major source for bone and adipocytes in adult bone marrow	Zhou et al. 2014
Nes-GFP Nes-CreER; Tomato Nes-GFP;Col2-Cre;Tomato	Nestin lineage cells in osteoblast and endothelial lineages	To identify the origin, heterogeneity, and fate of nestin-expressing cells during endochondral bone development	Nestin-expressing cells are associated with vasculature and encompass early cells in the osteoblast, stromal, and endothelial lineages	Ono et al. 2014a
Rainbow Actin-Cre-ERT	Bone, cartilage, and stromal tissue lineage-restricted clones	To identify mouse skeletal stem cells	Identification of mouse skeletal stem cell lineage of eight different cellular subpopulations with distinct skeletogenic properties	Chan et al. 2015
Grem1-CreERT; Tomato Grem1-CreERT; Tomato;Nes-GFP Grem1-CreERT; ZsGreen;Acta2-RFP Grem1-CreERT; R26-Confetti	Gremlin 1 (BMP antagonist) lineage cells in the bone marrow	To identify and determine the role of gremlin-1–positive MSCs in adult bone marrow	Gremlin-1–expressing cells are a source of osteoblasts, chondrocytes, and reticular marrow stromal cells in adult bone marrow	Worthley et al. 2015
Pthrp-mCherry Pthrp-CreER; Tomato	PTHrP lineage chondrocytes in the resting zone	To identity skeletal stem cells and how they are maintained in the growth plate	Skeletal stem cells are formed among PTHrP-positive chondrocytes within the resting zone of the postnatal growth plate	Mizuhashi et al. 2018

(continued)

Table 1 (continued)

Transgenic model	Cell lineage traced	Aim of the lineage tracing	Role of the lineage cells in bone marrow	Reference
Col1–GFP; Col2–Cre; Tomato Col1–GFP;Osx–Cre;Tomato Cxcl12GFP; Col2–Cre; Tomato Cxcl12–GFP; Osx–Cre; Tomato Acan-CreER; Tomato	Lineage cells defined by promoter/enhancer activities of Sox9, Col2 and Acan genes	To identify cells that supply osteoblasts and stromal cells in the metaphyses of growing bones	Cells defined by promoter/enhancer activities of genes such as Sox9, Col2, and Acan, encompass early mesenchymal progenitors that continue to become chondrocytes, osteoblasts, stromal cells, and adipocytes during endochondral bone development	Ono et al. 2014b
Hoxa11-CreERT2 Hoxa1-eGFP Hoxa11eGFP; Osx-CreER; Tomato Hoxa11eGFP; LepR-Cre; Tomato	Hoxa11 lineage cells	To understand the lineage relationships between Hox11 lineage cells and other genetically defined MSC populations	Hoxa11-lineage cells give rise to LepR-Cre and Osx-CreER lineage cells	Pineault et al. 2019

Abbreviations: Acan, aggrecan; αSMA, alpha smooth muscle actin; BMP, bone morphogenetic protein; Col2, collagen type II; CXCL12, C-X-C motif chemokine ligand 12; CXCR4, C-X-C chemokine receptor type 4; GFP, green fluorescent protein; HSC, hematopoietic stem cell; LepR, leptin receptor; Mx1, myxovirus resistance-1 promoter; Prx1, paired mesoderm homeobox protein 1; Pten, phosphatase and tensin homolog; PThrP, parathyroid hormone-related protein; RFP, red fluorescent protein; Sox9, SRY-Box 9; YFP, yellow fluorescent protein; zsGreen, Zoanthus species green

stromal tissue (Chan et al. 2015). They concluded that the $CD45^{-}/Ter119^{-}/Tie2^{-}/AlphaV^{+}/Thy^{-}/6C3^{-}/CD105^{-}/CD200^{+}$ cell population represented the putative mSSC population in postnatal skeletal tissues. Moreover, they investigated the transcriptome of the mSSCs and identified niche factors that were potent inducers of osteogenesis, which included bone morphogenetic protein 2 (BMP-2). Several specific combinations of recombinant mSSC niche factors were seen to activate the mSSC genetic programs in situ, even in nonskeletal tissues, which resulted in *de novo* formation of cartilage or bone and bone-marrow stroma (Chan et al. 2015).

Studies by Sugiyama and colleagues, Méndez-Ferrer and colleagues, and Pinho and colleagues focused on the identification of bone-marrow populations that mediate HSC maintenance and expansion (Sugiyama et al. 2006; Méndez-Ferrer et al. 2010; Pinho et al. 2013). Sugiyama and colleagues identified CXC chemokine ligand (CXCL)-12-abundant reticular (CAR) cells that resided in contact with

HSCs. Induced deletion of CXCR4, a receptor for CXCL12 in adult mice, resulted in severe reduction of HSC numbers, which thus indicated the essential role of CAR cells in the maintenance of the quiescent HSC pool (Sugiyama et al. 2006). Méndez-Ferrer and colleagues identified MSCs using nestin expression and demonstrated that these nestin$^+$ MSCs constituted an essential HSC niche component. Nestin$^+$ MSCs were spatially associated with HSCs and adrenergic nerve fibers, and they showed high expression of HSC maintenance genes. These genes and others triggered osteoblastic differentiation, which thus indicated a partnership between two distinct somatic stem-cell types in a unique niche in bone marrow (Méndez-Ferrer et al. 2010). Pinho and colleagues identified a PDGFRα$^+$/CD51$^+$ subset of nestin$^+$ cells that were enriched in major HSC maintenance genes, which provided further evidence that the niche activity co-segregates with MSC activity (Pinho et al. 2013).

Ono and colleagues and Mizoguchi and colleagues focused on the identification of the specific primary source of MSCs in fetal life (Mizoguchi et al. 2014; Ono et al. 2014a, b). Ono and colleagues identified cells that were defined by promoter/enhancer activities of genes associated with chondrocytes and their precursors, which included sex-determining region Y-box 9 (Sox9), collagen II (Col2), and aggrecan (Acan). These cells included early mesenchymal progenitors that continued to become chondrocytes, osteoblasts, stromal cells, and adipocytes during endochondral bone development (Ono et al. 2014b). In another study, Ono and colleagues identified distinct endothelial and nonendothelial nestin$^+$ cells in the embryonic perichondrium (Ono et al. 2014a). These nonendothelial nestin$^+$ cells were early cells of the osteoblast lineage, as immediate descendants from progenitors that required Indian hedgehog and Runx2 expression (Ono et al. 2014a). Mizoguchi and colleagues showed that osterix (Osx) defines the progenitors in fetal bone marrow that contribute to the nascent bone tissues and transient stromal cells that are replaced in adult bone marrow. The osterix-expressing cells also emerged later, that is, perinatally, to contribute to osteogenic lineages and long-lived bone-marrow stroma, and they had similar characteristics to nestin-GFP+ cells. In the adult bone marrow, osterix-expressing cells were restricted to the osteogenic lineage and were devoid of stromal contributions (Mizoguchi et al. 2014).

A number of studies have focused on the identification of the MSC populations involved in adult bone maintenance and regeneration (Méndez-Ferrer et al. 2010; Park et al. 2012; Grcevic et al. 2012; Zhou et al. 2014; Mizuhashi et al. 2018). Méndez-Ferrer and colleagues showed that in addition to forming a unique bone-marrow niche, adult nestin$^+$ MSCs contribute to skeletal remodeling, through differentiation into cartilage-forming and bone-forming cells (Méndez-Ferrer et al. 2010). Park and colleagues identified an Mx1$^+$ cell population that represented a highly dynamic and stress responsive stem/progenitor cell population with osteogenic fate-restricted potential, which supplied the high cell-replacement demands of the adult skeleton (Park et al. 2012). Grcevic and colleagues traced the progeny of α-smooth muscle actin (αSMA)-expressing perivascular cells to mature osteoblasts and osteocytes, to show that these cells contribute to the final stage of skeletal lineage differentiation (Grcevic et al. 2012). Zhou and colleagues showed that leptin receptor (LepR)-expressing cells arose postnatally and gave rise to most of the

osteogenic lineage cells and adipocytes that were formed in adult bone marrow (Zhou et al. 2014). Mizuhashi and colleagues showed that skeletal stem cells were formed among parathyroid hormone-related protein (PTHrP)$^+$ chondrocytes within the resting zone of the postnatal growth plate. The PTHrP$^+$ chondrocytes underwent hypertrophy and became osteoblasts and marrow stromal cells beneath the growth plate (Mizuhashi et al. 2018). In addition to showing that these MSC populations represented the mesenchymal progenitors of adult bone tissue remodeling, they also demonstrated that Lepr$^+$, Mx1$^+$, and αSMA$^+$ cells contributed to fracture repair (Park et al. 2012; Grcevic et al. 2012; Zhou et al. 2014).

Worthley and colleagues and Pineault and colleagues identified MSC populations in the earliest stages of skeletal development that self-renewed throughout the life of the animal (Worthley et al. 2015; Pineault et al. 2019). Here, Worthley and colleagues defined a population of osteochondroreticular stem cells in bone marrow that showed expression of the BMP antagonist gremlin 1 (Worthley et al. 2015). These cells self-renewed and generated osteoblasts, chondrocytes, and reticular marrow stromal cells, but not adipocytes. These osteochondroreticular cells were concentrated within the metaphysis of long bones and were not in the perisinusoidal space, and they were needed for bone development, bone remodeling, and fracture repair (Worthley et al. 2015). Pineault and colleagues identified cells with Hoxa11-lineage that gave rise to all of the skeletal lineages from embryonic stages throughout life. They also showed that these Hoxa11-lineage-positive cells gave rise to previously described MSC populations defined by LepR-Cre and Osx-CreER, which placed them upstream of these populations (Pineault et al. 2019).

Other studies have shown overlaps between various populations of MSCs. For instance, Nes-GFP$^+$ cells show high overlap with Lepr$^+$ perivascular cells (Pinho et al. 2013), which have been shown to be a major source of stem cell factor and CXCL12 in bone marrow (Ding et al. 2012).

2.2.2 In vitro Culture and Characterization of Mouse Bone-Marrow-Derived Mesenchymal Stromal Cells

Traditionally, isolation of MSCs has relied on their adherence to plastic. However, the cell populations obtained using such an approach represent a heterogeneous mixture of the various cell lineages that are present in the original tissue (Zhu et al. 2010; Houlihan et al. 2012). Thus, bone-marrow MSC cultures can also include osteoblasts, adipocytes, and stromal fibroblasts, as well as hematopoietic lineage cells, such as monocytes and osteoclasts. Given the low frequency of MSCs in the bone marrow, several methods have been developed to increase the yield and to isolate a more homogeneous population of bone-marrow MSCs (Morikawa et al. 2009; Zhu et al. 2010; Houlihan et al. 2012).

In general, mouse bone-marrow MSCs are isolated from the femur and/or tibia using the flushed (Méndez-Ferrer et al. 2010; Zhu et al. 2010; Houlihan et al. 2012; Grcevic et al. 2012; Pinho et al. 2013) or crushed (Morikawa et al. 2009; Houlihan et al. 2012) methods. For the flushed method, the bone marrow is flushed out of the bone-marrow cavity of the long bones using a syringe needle. For the crushed method, the long bones are gently broken up using a pestle and mortar, and the bone fragments are washed, digested with collagenase, and filtered, to obtain a cell

suspension equivalent to the flushed bone marrow (Morikawa et al. 2009; Houlihan et al. 2012). There is also the option of isolation of MSCs from bone tissue surfaces, where the bone-marrow cavities are flushed at least three times to thoroughly deplete the hematopoietic cells, followed by collagenase digestion (Guo et al. 2006; Zhu et al. 2010). In contrast with the crushed method, the digested bone fragments (rather than a cell suspension) are cultured in MSC medium until fibroblast-like cells are observed around the bone fragments, which is usually within 48 h (Zhu et al. 2010). Cells isolated from bone surfaces using this method were shown to have similar characteristics upon in vitro expansion to their counterparts from bone marrow, such as their immunophenotype, trilineage differentiation potential, and immunosuppression (Guo et al. 2006). Similarly, Sakaguchi and colleagues showed that after collagenase treatment, trabecular bone produces MSCs that upon in vitro expansion are virtually identical to their bone-marrow counterparts (Sakaguchi et al. 2009). The only observed differences here were in the expression of the surface markers CD54, that is, intercellular adhesion molecule 1 (ICAM-1), and CD106, that is, vascular cell adhesion protein 1 (VCAM-1), which might indicate the presence of endothelial and bone-lining cells in the MSC population obtained from trabecular bone.

In contrast, the crushed and flushed harvesting techniques were shown to result in different populations of bone-marrow MSCs (Morikawa et al. 2009; Houlihan et al. 2012; Pinho et al. 2013). For instance, Pinho and colleagues used the flushed method to harvest mouse bone marrow, and among the freshly isolated $CD45^-/Ter119^-/CD31^-$ cells they identified a subpopulation of $PDGFR\alpha^+/CD51^+$ bone-marrow stromal cells. This population characterized a large fraction of the $nestin^+$ cells and showed the highest fibroblastic CFUs and the most mesenspheres and self-renewal capacity after transplantation (Pinho et al. 2013). However, these cells did not express Sca-1, which had been indicated as a MSC maker in a previous study by Morikawa and colleagues, who identified $PDGFR\alpha^+$ and $Sca\text{-}1^+$ cells using the crushed isolation method, where the bone-marrow fraction was discarded (Morikawa et al. 2009).

Several chemical and physical methods have also been commonly used to enrich bone-marrow MSCs. In particular, these have included erythrocyte lysis, immunomagnetic depletion of $CD45^+$ cells, and fluorescence-activated sorting of the cells for positive MSCs markers (Méndez-Ferrer et al. 2010; Houlihan et al. 2012; Pinho et al. 2013). Through such methods, the yield and purity of MSCs can be improved. For instance, Houlihan and colleagues reported obtaining more than 10^7 $PDGFR\alpha^+/Sca\text{-}1^+$ MSCs from their original 5,000 cells seeded. When compared to traditional methods, this MSC subpopulation showed 120,000-fold higher fibroblastic CFU frequency than unfractionated bone marrow (Houlihan et al. 2012).

2.3 Studies of Bone-Marrow-Derived Mesenchymal Stromal Cells in the Human System

2.3.1 Human Bone-Marrow-Derived Mesenchymal Stromal Cells and Identification of Human Skeletal Stem Cells

While human bone-marrow aspirates and their derivatives are already being evaluated in clinical studies for the treatment of various skeletal disorders (see Sect. 4.3)

(Marolt Presen et al. 2019), in the human system, bone-marrow MSC populations remain poorly characterized compared to the above-discussed mouse models. In 2018, Chan and colleagues were the first to identify human SSCs (hSSCs) (Chan et al. 2018). They had previously identified mSSCs that gave rise exclusively to bone, cartilage, and marrow stroma (Chan et al. 2015) (see Sect. 2.2). As in the mouse system, a single-cell RNA sequencing approach was used with cells isolated from human fetal growth-plate samples, and a hSSC population was identified with a transcriptome analogous to that of their previously identified mSSC population. These hSSCs were characterized by the expression profile of podoplanin $(PDPN)^+$/$CD146^-$/$CD73^+$/$CD164^+$ (Chan et al. 2018), a finding that potentially provided the basis for enrichment of hSSCs for therapeutic use. The main obstacle for clinical translation, however, remains the scale-up of such cell populations to the cell numbers needed for regenerative medicine. Given the low frequency of hSSCs in adult bone marrow, this still represents a significant challenge.

As detailed above, the criteria for the in vitro definition of hMSCs date back to 2006, when the ISCT published their consensus of the minimal standard criteria, to promote more uniform characterization of MSCs, and to facilitate the exchange of data (Dominici et al. 2006). With the advances in the identification of hSSCs, it should be noted that the MSC consensus criteria encompass a broader and more heterogeneous population of cells that might not reproducibly show the desired regenerative capacities in vivo. For instance, a previous study has shown that MSC populations that express CD105, CD73, and CD90, and that are negative for CD45, CD34, CD14, and HLA class II, appear to be relatively rare and relatively heterogeneous in their surface marker expression levels and biological properties, and at various proportions, depending on the donor and tissue sources (Mendicino et al. 2014). Critically, variations were seen when the same donor and tissue source was used, as well as under different culture conditions (Mendicino et al. 2014). Thus, for the development of advanced MSC-based therapies for clinical trials, and with the recently defined hSSCs, further advances in the characterization of MSC-based products remain an unmet need.

Interestingly, bone-marrow MSCs, and perhaps hSSCs, can be affected by the (patho)physiological state of the donor. The in vitro properties of bone-marrow MSCs have been studied in health and disease (Murphy et al. 2002; Jones et al. 2010; Campbell et al. 2016), and most commonly in patients with age-related diseases, such as osteoarthritis and osteoporosis. These studies have shown that age and inflammation affect MSCs (reviewed by Jones and McGonagle (2008)), which results in higher baseline secretion of inflammatory mediators, and significant reduction of the osteogenic potential. Osteoporosis has been called the "obesity of bone," given that with aging, the composition of bone marrow shifts to adipogenesis, due to osteogenesis (Rosen and Bouxsein 2006). Secondary causes of osteoporosis are also associated with increased bone-marrow adiposity, such as diabetes mellitus, glucocorticoids use, and immobility (Rosen and Bouxsein 2006). In osteoarthritis, bone-marrow-derived MSCs were reported to have reduced chondrogenic potential (Murphy et al. 2002). Moreover, $CD45^-$/$CD271^+$ cell numbers were increased in bone-marrow lesions adjacent to cartilage defects and areas of osteochondral

angiogenesis. These MSCs showed lower proliferation and mineralization capacities in vitro (Campbell et al. 2016). Taken together, the potential for the use of bone-marrow MSCs for regenerative medicine needs further evaluation, and in particular for autologous therapies.

2.3.2 In Vitro Culture and Characterization of Human Bone-Marrow-Derived Mesenchymal Stromal Cells

As detailed in the ISCT consensus criteria (Dominici et al. 2006), bone-marrow MSCs are cultured on tissue-culture plastic and are characterized by expression of specific surface antigens, along with their tri-lineage differentiation potential (see Sect. 1; Fig. 2). Collection of human bone marrow from healthy individuals for research purposes is often limited for ethical reasons. However, human bone-marrow aspirates and isolated populations are available from commercial sources, and these are used commonly in research. Studies have also detailed the use of bone/bone-marrow tissue that remains as leftover material following surgical procedures, including femoral head, vertebral body, and iliac crest tissues (Chan et al. 2018;

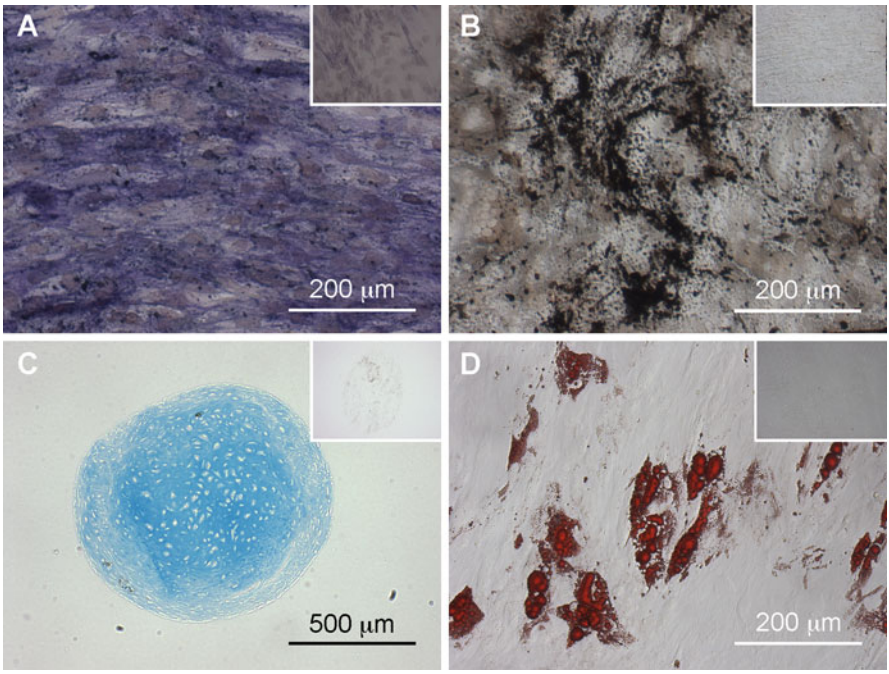


Fig. 2 Human bone-marrow-derived mesenchymal stromal cell characterization in vitro. Cultured bone-marrow MSCs are characterized for their potential to differentiate into osteogenic (**a**, **b**), chondrogenic (**c**), and adipogenic (**d**) lineages. Staining is for differentiated MSCs in monolayer cultures expressing alkaline phosphatase (**a**, purple) and mineralized matrix (**b**, black; von Kossa); pelleted differentiated MSCs in a glycosaminoglycans-rich matrix (**c**, blue; Alcian blue); and differentiated MSCs in monolayer cultures showing lipid vacuoles (**d**, red; Oil red O stain). Insets: negative staining of control cultures, grown without differentiation supplements

Herrmann et al. 2019) (Table 2). In a number of cases, cadaver tissues have also been used (Cavallo et al. 2011), with the appropriate ethical permission in place (as for tissues remaining after surgical procedures).

Tissue samples are collected, placed into physiological solution or cell-culture medium, and transported to the laboratory, where the tissue is further processed for MSC isolation and culture. If not aspirated, the bone marrow is scraped out or washed out of the bone spongiosa, broken or cut into fragments, collected, and either processed for mononuclear cell isolation or diluted in cell-culture medium and directly seeded into tissue-culture plates (Chan et al. 2018). Studies have reported higher yields of adherent cells and lower MSC losses after seeding of the whole bone marrow, in comparison to the mononuclear cell isolation methods (Ikebe and Suzuki 2014). However, adherent cell populations obtained from whole bone marrow are more heterogeneous, as they consist not only of MSCs, but also of hematopoietic cells at different differentiation/commitment stages, endothelial cells, and endothelial progenitor cells. To obtain a more homogeneous MSC population in primary cultures, density gradient centrifugation is commonly used (Pittenger et al. 1999), and in some studies, lysis of the red blood cells (Lee et al. 2003). More purified MSC populations can be obtained from bone-marrow samples using fluorescence-activated cell sorting or immunomagnetic bead sorting (Pountos et al. 2007). These techniques are limited because of the additional work, potential cell damage and/or loss during processing, additional cost, and lack of MSC-specific surface markers (Ikebe and Suzuki 2014). Examples of cell isolation, seeding, and cultivation protocols to establish primary cultures of human bone-marrow MSCs are given in Table 2.

When primary cultures of bone-marrow MSCs reach confluence, the cells are typically sub-cultured using standard trypsinization procedures. Nonadherent contaminating cells are gradually removed from the cultures through medium changes and passaging. In vitro studies commonly report using bone-marrow MSCs up to the fifth passage for experiments. With extended passaging, MSCs show a decline in differentiation and proliferation potential, and enter replicative senescence (Solchaga et al. 2010; Schallmoser et al. 2010; de Peppo et al. 2010; Jones et al. 2010; Cheng et al. 2011). The numbers of cells obtained in primary cultures, their proliferation and differentiation potential, and the maximum number of population doublings can vary greatly between individual donors and sites of tissue harvest (Pittenger et al. 1999; Herrmann et al. 2019) and might decline with advanced chronological age of the donor (Stolzing et al. 2008). Of note, $CD73^{+}$/STRO-1^{+}/$CD105^{+}$/$CD34^{-}$/$CD45^{-}$/$CD144^{-}$ cells, isolated from collagenase-digested trabecular bone surfaces, are similar to bone-marrow MSCs in terms of their proliferation and differentiation (Tuli et al. 2003).

Standard two-dimensional culturing on tissue-culture plastic represents an artificial environment that does not recapitulate the native tissue environment of bone-marrow MSCs. Modifications to the culture procedures have mainly been through supplementation of the growth medium with specific inductive factors (e.g., basic fibroblast growth factor (bFGF) Martin et al. 1997; Solchaga et al. 2005; Solchaga et al. 2010; Di Maggio et al. 2012) and cultivation on protein substrates or cell-derived extracellular matrices. These have been shown to enhance bone-marrow MSC growth and clonogenicity and to maintain their differentiation potential (Mauney et al. 2005, 2006; Lai et al. 2010). However, cultivation on nonplastic

Table 2 Examples of human bone-marrow-derived mesenchymal stromal cell isolation, seeding, and primary culture procedures. All of these systems were cultured on tissue-culture plastic

	Donor information			Seeding	Culture		
Tissue source	Gender	Age (years)	Isolation method	density	Medium	Medium supplements	Reference
Bone-marrow aspirate from iliac crest	1 female, 2 males	na	Density gradient centrifugation	2.0×10^5 NC/cm^2	DMEM	10% fetal bovine serum (lot selected)	Pittenger et al. 1999
Bone-marrow aspirate from iliac crest	6 females	32–51	Density gradient centrifugation	1.0×10^5 NC/cm^2	DMEM	10% fetal bovine serum (lot selected), NEAA, L-glutamine, sodium pyruvate, HEPES, penicillin/streptomycin, without/with bFGF, without/with dexamethasone	Frank et al. 2002
Bone-marrow aspirate from iliac crest	na	3–49	Whole bone-marrow seeding	$2–5 \times 10^6$ MNC/100-mm dish	Coon's modified Ham's F-12	10% fetal calf serum	Bianchi et al. 2003
Bone-marrow aspirate from femoral head	2 males	58, 72	Red blood cell lysis, whole bone-marrow seeding	na	DMEM	10% fetal bovine serum, penicillin/streptomycin	Lee et al. 2003
Femoral head	na	50–70	Whole bone-marrow seeding	na	DMEM	10% fetal bovine serum, penicillin/streptomycin	Li et al. 2005
Bone-marrow aspirate	7 donors	na	Density gradient centrifugation	1.8×10^5 NC/cm^2	DMEM	10% fetal bovine serum (lot selected)	Solchaga et al. 2005
Bone-marrow aspirate	2 females, 3 males	13, 30, 36, 47, 58	Whole bone-marrow seeding	na	α-MEM	10% pHPL, heparin, L-glutamine	Schallmoser et al. 2008
Femoral head	3 patients		Whole bone-marrow seeding	na	DMEM	10% fetal bovine, penicillin/streptomycin	García Quiroz et al. 2008

(continued)

Table 2 (continued)

Tissue source	Donor information		Isolation method	Seeding	Culture	Medium supplements	Reference
	Gender	Age (years)		density	Medium		
Bone-marrow aspirate from iliac crest, femoral head	4 females, 7 males	57–79	Density gradient centrifugation	1.0×10^6 MNC/cm^2	DMEM	10% fetal calf serum (lot selected)	Sanchez-Guijo et al. 2009
Bone-marrow aspirate	1 female, 2 males	9, 27, 36	Whole bone-marrow seeding	$0.6–1.0 \times 10^4$ MNC/cm^2	α-MEM	10% fetal bovine serum or 10% pHPL, HEPES, L-glutamine, penicillin/streptomycin	Schallmoser et al. 2010
Bone-marrow aspirate from iliac crest	42 donors	2–61	Enzymatic digestion of	na	Commercial medium	na	Jones et al. 2010
Commercial bone-marrow MNCs	22 donors	19–39	Dissected bone tissue,				
Trabecular bone from pelvic area	20 patients	17–86	Immunomagnetic bead sorting				
Bone-marrow aspirate from pelvic area	6 patients	25–61					
Femoral head	51 patients	43–91					
Bone-marrow aspirate, commercial, iliac crest	21 donors		Whole bone-marrow seeding	1.2×10^4 MNC/cm^2; 5×10^4 WBC/cm^2	α-MEM	5% or 10% platelet lysate, heparin	Fekete et al. 2012

Bone-marrow aspirate, commercial, iliac crest	3 males	Late 20s, 22, 27	Density gradient centrifugation	na	DMEM	Fetal bovine serum, penicillin/ streptomycin, bFGF	Hung et al. 2015
Bone-marrow aspirate from vertebral body	3 females, 7 males	39–89	Density gradient centrifugation	5×10^4 MNC/cm^2	α-MEM	10% fetal calf serum, penicillin/ streptomycin, bFGF	Herrmann et al. 2019
Bone-marrow aspirate from iliac crest	3 females, 5 males	14–88					
Bone-marrow aspirate from femoral head	4 females, 11 males	46–91					

Abbreviations: α-MEM, α-modified minimum essential medium; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-AA2P, L-ascorbic acid 2-phosphate; MNCs, mononuclear cells; na, data not available; NC, nucleated cells; NEAA, nonessential amino acids; pHPL, pooled human platelet lysate; WBC, white blood cells

substrates violates one of the three consensus criteria for the definition of bone-marrow MSCs (Dominici et al. 2006). In light of the recent identification of hSSCs (Chan et al. 2018), the in vitro conditions that can potentially support hSSC maintenance and expansion for research and therapeutic purposes need to be further investigated.

Characterization of the properties of bone-marrow MSC involves the quantification of the expression of specific surface antigens using flow cytometry/fluorescence-activated cell sorting, as well as testing for their osteogenic, chondrogenic, and adipogenic differentiation potential (Fig. 2; Table 3). For osteogenic differentiation, induction in two-dimensional monolayer cultures or in three-dimensional pellet cultures can be used. Osteogenic differentiation media contain a combination of supplements, which are most commonly ascorbic acid or ascorbic acid 2-phosphate, β-glycerophosphate, and dexamethasone, and in some cases vitamin D and BMP-2 (Jaiswal et al. 1997; Marolt et al. 2006). For chondrogenesis evaluation, small three-dimensional cultures are most frequently used, to allow a cell condensation step that mimics the chondrogenic process during skeletal development. Chondrogenic pellets are prepared using brief centrifugation of MSC suspensions, followed by incubation of the resulting cell pellet in induction medium (Johnstone et al. 1998; Solchaga et al. 2010). Chondrogenic micromass cultures are prepared by seeding droplets of dense MSC suspensions into culture wells, an incubation to allow cell condensation, followed by addition of the induction medium (Chan et al. 2018). Chondrogenic induction media are mostly serum-free and contain a combination of supplements, which usually include insulin, transferrin, selenous acid, pyruvate, ascorbic acid 2-phosphate, dexamethasone, and transforming growth factor beta-1 (TGFβ-1) or TGFβ-3 (Johnstone et al. 1998; Solchaga et al. 2005; Bhumiratana et al. 2014). Adipogenic differentiation potential is commonly evaluated in two-dimensional monolayer cultures (Pittenger et al. 1999). Here, the cells are grown to confluence and exposed to adipogenic induction/maintenance medium, which contains a combination of supplements that generally includes dexamethasone, methyl-isobutylxanthine, insulin, and indomethacin. At various times during differentiation, which is commonly between 1 and 6 weeks of culture, the phenotype development is evaluated using a combination of histological, biochemical, and molecular assays (Table 3).

3 Bone-Marrow-Derived Mesenchymal Stromal Cells in Bone Tissue Engineering and Bone Regeneration

3.1 Biology of Bone Healing and the Rationale for Bone Tissue Engineering

Bone undergoes constant remodeling to accommodate dynamic biomechanical stresses. Given optimal conditions, bone has the remarkable potential to self-repair, to such an extent that the regenerated tissue cannot be distinguished from its state prior to injury in either form or function (Rosen 2013). However, this regenerative

Table 3 Methods commonly used to characterize human bone-marrow-derived mesenchymal stromal cells

Consensus criterion	Method	Specific markers
Plastic adherent growth	Microscopy	Fibroblast-like morphology
Surface antigen expression	Flow cytometry, fluorescence-activated cell sorting	Positive: >95% cells expressing antigens CD73, CD90, CD105 Negative: <2% cells expressing antigens CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR
Osteogenic differentiation	Histological staining	Alkaline phosphatase activity, collagen staining with Picrosirius Red, mineralization staining with von Kossa or Alizarin Red S
	Immunofluorescence, immunohistochemistry	Collagen type I, osteopontin, osteocalcin, bone sialoprotein
	Biochemical	Proliferation, DNA, alkaline phosphatase activity, calcium
	ELISA	Osteopontin, osteocalcin
	Gene expression	Alkaline phosphatase, collagen type I, Runt-related transcription factor 2 (Runx2), osteopontin, osteocalcin, bone sialoprotein
Chondrogenic differentiation	Histological staining	Glycosaminoglycan staining with Alcian Blue, Toluidine Blue or Safranin O
	Immunofluorescence, Immunohistochemistry	Collagen type I, II, X, aggrecan, versican
	Biochemical	Proliferation, DNA, collagen, glycosaminoglycans
	Gene expression	Collagen type I, II and IX, aggrecan, versican, Sry-box transcription factor 9 (SOX9)
Adipogenic differentiation	Histological staining	Lipid vacuoles with oil red O or Nile red
	Biochemical	Proliferation, DNA
	Gene expression	Lipoprotein lipase (LPL), fatty acid binding protein-4 (FABP4), complement factor D (CFD; adipsin), peroxisome proliferator activated receptor gamma (PPARG), adiponectin, C1Q, collagen domain containing (ADIPOQ)

activity declines with increasing age (Boskey 2013; Gibon et al. 2016) and is limited in bone defects greater than a certain size (termed critical-size defects). A common exacerbating condition is osteoporosis, a disease that occurs when there is an impairment in the bone remodeling processes, and this, in turn, increases the fracture risk, particularly in the elderly.

Fracture healing occurs either by the direct intramembranous pathway or the indirect pathway, and it represents a complex biological process that is intertwined with the innate immune system. Furthermore, direct (i.e., primary) fracture healing

requires anatomical reduction and rigid stabilization, to thus minimize the fracture gap and interfragmentary motion. Such an approach allows the bone to heal by direct regeneration of anatomical lamellar bone followed by Haversian systems, without the need for remodeling to occur. When a slightly larger gap exists between the bone fragments, gap healing occurs, in which voids are filled with direct deposition of intramembranous woven bone (Einhorn and Gerstenfeld 2015; Loi et al. 2016). Complete rigidity is not possible for most fractures treated by splinting, intramedullary nailing, or external fixing methods. In these scenarios, secondary fracture healing involves intramembranous and subsequent endochondral ossification (Fig. 3) (Loi et al. 2016). Establishment of the normal integrity of fractured bone can take months or even years to complete (Loi et al. 2016), with up to 10% of all bone fractures prone to delayed bone union, progress to nonunion, or development of a pseudoarthrosis (Boskey 2013). Successful fracture healing is dependent on several interlinking factors that affect the mechanical stability of the fracture, the influx of osteogenic and inflammatory cells, growth factors and chemotactic mediators, and adequate vasculature (Fig. 3).

The main treatment for bone defects remains bone grafting (Gibbs et al. 2014). A bone graft is implanted material that can be used alone or in combination with other materials, which promotes bone healing through osteoinduction, osteoconduction, and osteogenesis. Tissue viability, defect size, graft size, shape and volume, biomechanical properties, graft handling, and cost issues all influence the selection of an ideal bone graft in any particular clinical situation. This can be in the form of an autograft (i.e., harvested from the patient), an allograft (i.e., obtained from a donor),

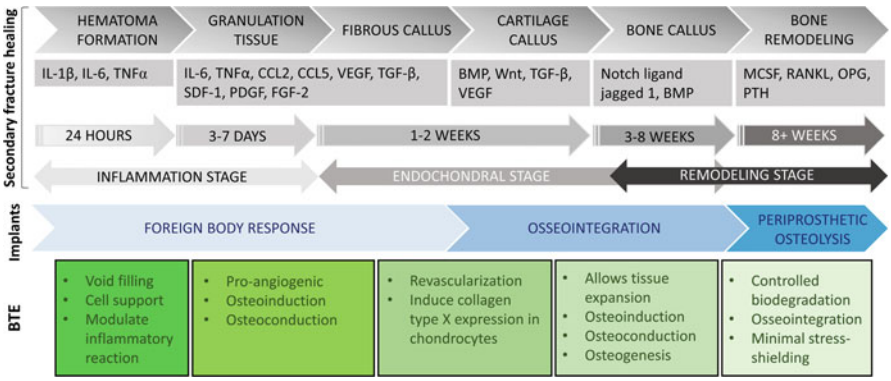


Fig. 3 Stages of secondary fracture healing compared to bone healing with implants, and the key properties of bone tissue engineering (BTE) constructs that can support each stage. (Adapted from Einhorn and Gerstenfeld 2015; Loi et al. 2016; Winkler et al. 2018). BMP, bone morphogenetic protein; CCL, C-C motif chemokine ligand; IL-1β, interleukin-1β; IL-6, interleukin-6; FGF-2, fibroblast growth factor 2; MCSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; PDGF, platelet-derived endothelial growth factor; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor kappa-B ligand; SDF-1, stromal cell-derived factor 1; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; Wnt, wingless-related integration site

Table 4 Advantages and disadvantages of bone grafts. (Adapted from Tang et al. 2016)

Graft type	Advantages	Disadvantages
Autograft	Osteogenic, osteoconductive, osteoinductive	Patient morbidity, pain and infection at donor site, visceral injury during harvesting, reduced vascularization, limited quantity from patient
Allograft or xenograft	Osteoconductive, osteoinductive, high availability, no donor site morbidity	Reduced osteogenic potential, relatively higher rejection risk, risk of disease transmission, cost
Engineered substitutes	Growth-factor and stem-cell constructs to improve osteogenicity and graft incorporation, can match the defect site using three-dimensional biofabrication techniques, no donor site morbidity	Osteogenicity limited by material porosity, variable biodegradability of different materials, limited mechanical properties, poor neovascularization and unknown immune response, cost

a xenograft (i.e., obtained from an animal), or the use of an engineered synthetic or biomaterial substitute (Gibbs et al. 2014). Over two million bone grafts are carried out every year around the world, but this approach is not without disadvantages (see Table 4, for more details).

3.2 Current Bone Tissue Engineering and Bone Regeneration Strategies

The approach of using biomaterials, cells, and/or signaling factors to repair bone defects or to generate bone tissue substitutes has been termed "bone tissue engineering" (BTE). Although the complex processes that drive and regulate the reparative capacity of bone have yet to be fully elucidated, current BTE strategies have begun to adopt an increasingly biomimetic approach (Fig. 4). Such an approach seeks to facilitate bone regeneration by providing a suitably conducive microenvironment that synergistically accelerates the native regenerative processes. While this rationale might appear simple, it has proven to be extremely difficult to achieve; indeed, BTE has yet to truly achieve clinical translation. Classical strategies in BTE and regeneration research, as well as the role of biofabrication in the evolution of BTE, are discussed in the following sections.

3.2.1 Biomaterial-Driven Strategies for Bone Tissue Engineering and Bone Regeneration

The scaffold architecture in bone tissue engineering classically consists of a primary solid support structure, with an open (and ideally interconnected) pore network. Matrices are typically hydrogel-based and are often less porous. Both forms must have several appropriate bio-physicochemical attributes, such as mechanical strength, stiffness, porosity, biodegradability, and surface chemistry for tissue formation, as well as having the capability to withstand and respond to mechanical stress (Fig. 4) (Zadpoor 2015). *In silico* and multimodal imaging and tracking

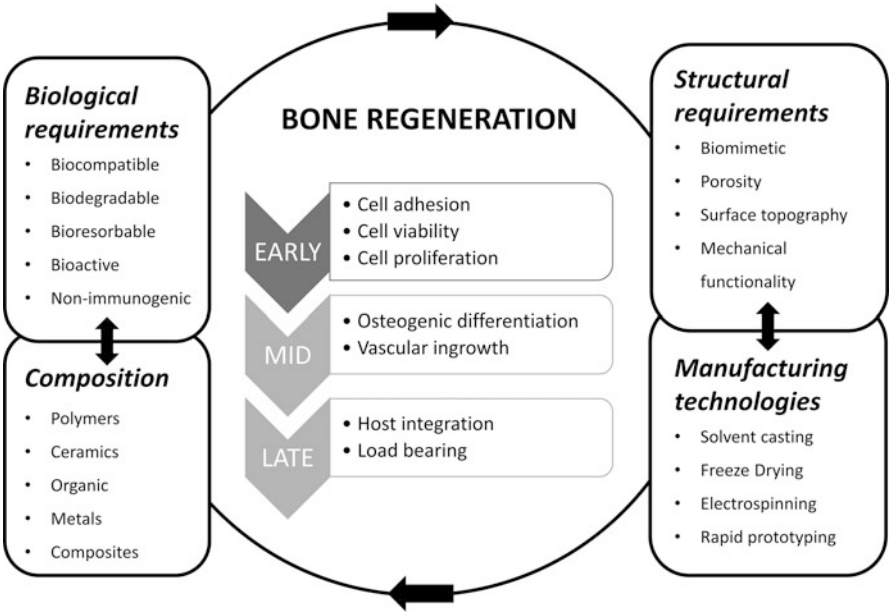


Fig. 4 The interlinked factors in contemporary bone regeneration strategies. (Adapted from Roseti et al. 2017)

approaches are being developed to help identify optimal scaffold design parameters for BTE (Tang et al. 2016; Santiesteban et al. 2016; Uth et al. 2017). Conventional methods that are used to fabricate BTE scaffolds suffer from variations in the control of pore size and geometry, interconnectivity, and limited scalability (Fig. 4) (Tasoglu and Demirci 2013). Therefore, scaffold design and manufacturing techniques used in BTE are key determinants of the biomechanical efficacy of the scaffold in vivo. These, in turn, rely on the properties of the chosen biomaterial(s).

Bone tissue engineering scaffolds are typically composed of bioceramics, metals, self-assembly peptides, and/or synthetic or natural polymers (Leijten et al. 2015; Ho-Shui-Ling et al. 2018). Composite scaffolds are increasingly used to overcome specific disadvantages of each individual biomaterial. Several combinations of biomaterials without and with cells have been investigated for their potential use in BTE using in vitro and/or in vivo methods (Oryan et al. 2014; Turnbull et al. 2018). Surface modification techniques, such as surface coating and electrochemical deposition or oxidization, can be used to enhance biomaterial biocompatibility and osteoinductive potential (Fernandez-Yague et al. 2015). These postmanufacturing processes can introduce physicochemical alterations to surface roughness (and thereby, topography) and/or surface wettability. However, despite some success in vitro, there have been few in vivo studies that have shown significantly enhanced osseointegration (Fernandez-Yague et al. 2015). This is reflected by the paucity of biomaterials that have achieved clinical translation, and their limited efficacy (Ho-Shui-Ling et al. 2018). There is also no clear consensus as to which

biomaterial(s), scaffold design, or manufacturing approach is ideal for bone regeneration purposes.

Biomaterial microengineering can be used to define the necessary biophysical and bioactive signals within stem-cell niches that are required for guided cell remodeling of tissues, and for generation of personalized tissue-engineered constructs from autologous cells (reviewed by Kobolak et al. (2016)). Investigations into the mechanisms behind human MSC commitment have revealed that the local tissue microenvironment regulates the commitment of stem cells to different lineages. Not only biochemical, but also mechanical and mechano-chemical cues affect the tissue microenvironment. In their seminal studies, McBeth et al. showed that cell shape regulates commitment of human MSCs to adipocyte or osteoblast fate (McBeath et al. 2004). Human MSCs that adhered, flattened, and spread underwent osteogenesis, while those that did not spread, and instead remained rounded, became adipocytes. The underlying mechanism was shown to be the modulation of endogenous RhoA activity, where adipogenesis was induced by expression of dominant-negative RhoA, while constitutively active RhoA resulted in osteogenesis. Such approaches might represent a stepping-stone toward the development of advanced bioactive scaffolds, and an enhancement of the activity of transplanted or endogenous cells.

3.2.2 Biomimetic-Driven Strategies for Bone Tissue Engineering and Bone Regeneration

In vivo, biochemical signals secreted at local injury sites or in areas undergoing bone remodeling can trigger migration of progenitors and inflammatory cells (i.e., in bone injury), or activation of osteoblasts and osteoclasts (i.e., in bone remodeling) (Kanczler and Oreffo 2008; Siddiqui and Partridge 2016). Over the past decade, poor in vivo osseointegration of cell-free scaffolds has led to their integrated use with cells (see Sect. 3.2.4.) and bioactive molecules (reviewed by Tang et al. (2016)). Such an approach harnesses the innate regenerative properties. These biomimetic scaffolds have been termed "smart scaffolds." Studies have investigated the efficacy of using growth factors (Sukul et al. 2015), synthetic peptides (Kesireddy and Kasper 2016), small molecules (Kim et al. 2017), and nucleic acids (Raftery et al. 2018) (Table 5). Recent studies have also included the use of the paracrine effect of

Table 5 Delivery strategies for biomolecules in bone tissue engineering. (Adapted from Dang et al. 2018)

Biomolecule type	Example	Delivery strategy
Nucleic acid	miRNA, siRNA	Intracellular and intranuclear preprogramming
Small molecule	Bisphosphonates	Surface adsorption, sustained release
Growth factor	BMP-2, VEGF	Surface presentation, controlled release
Endocrine signal	PTH, Vitamin D_3, estradiol	Time-controlled release
Inorganic biomaterial	Bioceramic, bioglass	Surface adsorption/deposition

the secretome of MSCs or stromal cells (which is shuttled through the combination of released soluble factors and extracellular vesicles, or is embedded within extracellular matrix molecules) to target osteoinduction and the inflammatory and immunity pathways involved in bone regeneration (Dang et al. 2018; Haumer et al. 2018; Marolt Presen et al. 2019) (see Sect. 5 for more details).

Different delivery systems are needed for different types of biomolecules to achieve optimal therapeutic effects. Table 5 lists the current delivery systems used in BTE (Dang et al. 2018). Growth factors have reached clinical translation, although of note most studies use growth factors at supraphysiological doses (i.e., mg per mL, rather than ng per mL), which can result in adverse effects such as ectopic bone formation, antibody development, and, potentially, carcinogenesis (Leijten et al. 2015). Furthermore, the actual dose of growth factors delivered by constructs in vivo when used in skeletal defect studies is unknown (Santo et al. 2013), and there remains no consensus as to the appropriate doses of growth factors or biomolecule combinations for BTE, with a wide range in use (Gothard et al. 2014). Only a few synthetic grafts that contain bioactive molecules are commercially available for surgical use in certain countries (Ho-Shui-Ling et al. 2018).

3.2.3 Biofabrication for Bone Regeneration

Advances in additive manufacturing technology have allowed tissue engineers to micro-engineer scaffold nanotopographies to aid, and to even enhance, cell attachment, growth, interactions, and migration (Dalby et al. 2014). Additive manufacturing techniques were initially developed in the mid-1980s, and they have been increasingly used to manufacture three-dimensional (3D) artificial implants using a myriad of existing biomaterials in controlled, reproducible manners that meet good manufacturing practice (GMP) standards (Gibbs et al. 2014; Fernandez-Yague et al. 2015). Another key advantage of additive manufacturing is its use to scale-up scaffold fabrication. The use of additive manufacturing in the fabrication of BTE constructs has also driven advances in additive manufacturing itself (Tang et al. 2016). Table 6 summarizes the most commonly investigated 3D printing methods that can be used for bone biofabrication today.

Additive manufacturing has not only been used for concomitant spatial printing of different cell types on 3D scaffolds to generate complex tissues (Kang et al. 2016; Keriquel et al. 2017), but also for incorporation of biomolecules within a matrix or within the scaffold itself during the printing process, to allow controlled drug delivery and/or gradient release (Tarafder and Lee 2017; Koons and Mikos 2019). This process of creating integrated cell–biomaterial–biomolecule constructs has been termed biofabrication, and it is typically divided into two categories based on the workflow pattern: the “top-down” and “bottom-up” approaches. The survival of the cells and their functionality postprinting is central to these approaches, to allow bone remodeling through external and internal stimuli (Fedorovich et al. 2011). The tunable ability to seed cells and biomolecules in 3D space with precision and in a user-controlled manner gives biofabrication an advantage over conventional techniques (Tang et al. 2016).

Table 6 Common three-dimensional printing methods used in bone biofabrication. (Adapted from Tang et al. 2016)

Method	Advantages	Limitations
Microextrusion deposition	Good precision and microscale resolution, wide variety of biomaterials, capable of printing physical or compositional gradients, cell and bioactive factor bioprinting, potential for up-scalable construct fabrication	Cell survival postbioprinting dependent on bioink properties, printing temperature, and build time, often requires use of support materials to fabricate porous constructs, increasing build time, low to moderate cost
Laser-assisted bioprinting	Nanoscale precision and printing under ambient conditions, capable of printing multiple physical or compositional gradients, simultaneous cell and bioactive factor bioprinting	Remains very cost-prohibitive, limited up-scalability, slow process
Inkjet-based cell printing	Low cost, capable of printing gradients and simultaneous cell and bioactive factor bioprinting, with high cell viability postbioprinting, relatively rapid fabrication	Limited biomaterial choice due to bioink printing requirements, poor resolution, limited scalability

Biofabrication generates a customized 3D construct that is a closer fit to the defect (in comparison to traditional bone grafts), which thereby reduces the risk of failure of the engraftment or repair. Use of autologous cells can also lower the risk of rejection. However, current biofabrication techniques have limitations. The choice of biomaterial for biofabrication is currently limited by the additive manufacturing method used. Additive manufacturing methods, such as stereolithography, require postprocessing procedures that are cytotoxic, while laser sintering can cause thermodegradation of the biomaterial, with the consequent loss of its precise microstructure, which in turn affects the material porosity and cell viability (Melchels et al. 2012; Gibbs et al. 2014). No single biofabrication approach has yet been defined as ideal for BTE purposes, with varying process protocols, limited up-scalability, and questionable cell viability post-bioprinting thus far restricting much progress in the field (Tang et al. 2016).

3.2.4 Cell-Based Strategies for Bone Tissue Engineering and Bone Regeneration

The cell source used for BTE and bone regeneration is crucial and depends on the specific experimental or clinical situation. Specific advantages and disadvantages of cell sources that are under investigation for BTE are presented in Table 7. Bone-marrow-derived stromal cells are the most frequently used cell source for BTE given their various advantages, which include ease of acquisition and cultivation, and extensive characterization (Dawson et al. 2014; Robey et al. 2015). Adipose-tissue-derived stromal cells have been proposed as a viable alternative due to their reported osteogenic capability in vitro, and their relative abundance and survival in low oxygen and low glucose environments (Szpalski et al. 2012). Such resilience is advantageous, particularly when the blood supply is limited. There has also been

Table 7 Cell types used in bone tissue engineering and bone regeneration. (Adapted from Szpalski et al. 2012; Liu et al. 2015; Csobonyeiova et al. 2017)

Cell type	Differentiation stage	Potential for bone tissue engineering	Advantages	Disadvantages
Bone-marrow-derived stem cells	Multipotent	Osteogenic, support neovascularization	Relatively easy acquisition, ease of culture, extensively characterized, patient specific	Donor morbidity, limited proliferative potential, properties depend on age and health of the donor
Umbilical vein stem cells	Multipotent	Osteogenic	Accessible with no donor morbidity, high proliferation, ease of culture	Limited characterization
Dental pulp, gingival stem cells	Multipotent	Osteogenic	Ease of acquisition, abundant	Limited characterization
Adipose-derived stem cells	Multipotent	Osteogenic, potential for neovascularization	Ease of acquisition, abundant, extensively characterized, ease of culture, patient specific	Donor morbidity
Endothelial progenitor cells (EPC), specifically endothelial colony-forming cells (ECFC)	Lineage-directed	Potential for neovascularization, support osteogenic differentiation	Ease of acquisition (peripheral blood, umbilical cord blood), minimal donor morbidity	Not multipotent, limited proliferation capacity, lack of standard isolation/cultivation procedures limits functionality assessment
Human umbilical vein endothelial cells	Differentiated	Potential for neovascularization	Accessible with no donor morbidity, can be co-seeded with bone-marrow-derived stem cells	Not multipotent
Human induced pluripotent stem cells	Pluripotent, differentiated	Any bone-regeneration related lineage	Ease of acquisition with established reprogramming protocols, unlimited self-renewal, existing lineage differentiation protocols, minimal or no donor morbidity, patient specific	Variable cell reprogramming efficiency, necessary induction into high quality progenitor cells prior to transplantation, possible risk of tumor formation

recent interest in using oral-cavity MSCs, perinatal-tissue-derived MSCs, and human induced pluripotent stem-cell-derived MSCs for BTE (Szpalski et al. 2012; Csobonyeiova et al. 2017).

Cell-based BTE encompasses various strategies, which range from cell seeding/mixing with 3D scaffolds directly prior to implantation (Léotot et al. 2015), to in vitro cultivation of cell/scaffold constructs to support new bony tissue growth and maturation; these involve cell proliferation and specific matrix deposition and increased mechanical properties (Frohlich et al. 2008; Ma et al. 2014; Marolt Presen et al. 2019). In a less common approach, cells and inductive factors have been combined with a scaffold and transplanted into an ectopic site in muscle, to use the body as a "bioreactor." This can allow the growth of large bone grafts, which can then be harvested through secondary surgery for bone-defect reconstruction (Warnke et al. 2004).

Bone-marrow MSCs are commonly expanded for several passages before BTE protocols, and they can be seeded on scaffolds directly or preinduced into the osteogenic lineage (Beloti et al. 2012). Cell seeding of porous scaffolds has been achieved using direct loading of cell suspensions into scaffolds (Marolt et al. 2006; Grayson et al. 2011). Alternatively, bioreactor-assisted dynamic seeding by the mixing or perfusion of cell suspensions into scaffolds has been used to increase the seeding efficiency (Hofmann et al. 2007; Timmins et al. 2007; Melke et al. 2020). The initial number of seeded cells has been shown to influence the development of the engineered bone (Lode et al. 2008; Wu et al. 2015).

For cultivation, standard static cultures have been used in numerous studies to evaluate the different types of scaffolds, signaling factors, signaling pathways, and cell types used for BTE. However, static culture limits new tissue development to less than 0.5 mm in thickness due to the mass transport limitations of diffusion, and to a batch feeding regime. To support the development of thicker tissue, cultures under dynamic culture systems – bioreactors – have been examined extensively, which have included rotating vessel bioreactors (Marolt et al. 2006), spinner bioreactors (Hofmann et al. 2007), and perfusion bioreactors (Grayson et al. 2011). Perfusion systems have shown great promise for the support of bone-like tissue formation, as these provide enhanced mass transport within the cultured constructs, and biophysical stimulation of the cells by hydrodynamic shear (Sikavitsas et al. 2003; Grayson et al. 2011; Bhumiratana et al. 2014). Furthermore, anatomical bioreactor systems that can support the development of custom-shaped bone grafts have been constructed with a view to clinical translation. These bioreactors supported the formation of bone-like grafts from bone-marrow MSCs that were several centimeters in size, and which are appropriate for evaluation in preclinical studies using large animals (Grayson et al. 2010). However, most in vitro BTE strategies have significant challenges for their clinical translation, mainly due to increased protocol complexity, culture duration, regulatory requirements, and costs of translation. Thus, there have only been a few clinical studies to date that have evaluated BTE approaches for the improvement of bone regeneration (see Sect. 4.3).

3.3 Vascularization: The Key Hurdle Toward Achieving Clinical Translation

An important unresolved technical hurdle in BTE that has hindered the generation of clinically successful constructs to date is vascularization. Bone is a metabolically active tissue that is supplied by an intraosseous vasculature, with osteocytes located only up to 100 μm from an intact capillary (Goggin et al. 2016). Spontaneous neovascularization upon implantation of a bone graft is triggered by inflammation. These capillary networks are transient and regress within a few weeks. Host-derived neovascularization of an implant is slow (i.e., at <1 mm a day) and is insufficient for constructs that are of a clinically relevant size, due to the limited degree of penetration into the implant to form functional capillaries. As complex engineered 3D constructs of clinically relevant size cannot be sustained by diffusion alone, the co-creation of a functional vascular network is considered necessary to ensure nutrient supply and, equally critical, waste removal, throughout the construct (Nguyen et al. 2012).

Bone tissue engineering currently focuses on co-induction of osteogenesis and angiogenesis through cell co-cultures, with the incorporation of growth factors into scaffolds, or using re-seeded decellularized tissues to generate microvasculature and bone regeneration. This approach is based on tight coupling of angiogenesis with osteogenesis in endochondral bone formation and bone fracture repair processes. The effects on osteogenesis and angiogenesis of the use of two cell types have garnered much interest, with increasing evidence supporting the use of bone-marrow stromal cells in the generation of blood vessels, particularly when seeded onto a scaffold with endothelial cells (Fedorovich et al. 2010). However, the use of co-cultures remains a limited approach in BTE, as few groups have managed to show improved bone formation using this approach (Liu et al. 2015; Unger et al. 2015). Liu and colleagues demonstrated that stimulated co-cultures of human fetal bone-marrow-derived MSCs and umbilical-cord-blood endothelial progenitor cells can result in earlier vessel infiltration and increased ectopic bone formation in vivo (Liu et al. 2013). Ko and colleagues also demonstrated vascularized bone regeneration through the seeding of decellularized tendons with human adipose-tissue-derived stem cells and human cord-blood-derived endothelial progenitor cells (Ko et al. 2016). Vascular endothelial growth factor (VEGF) is known to induce vascularization in endothelial cells, and it also binds to osteoblast receptors to promote bone repair, while BMP-2 directly induces osteoblast differentiation and promotes angiogenesis through induction of VEGF secretion from osteoblasts (Gothard et al. 2014). This synergistic effect has been used in several studies to improve osteogenic and angiogenic outcomes. For example, bioprinted porous scaffolds that incorporate VEGF and BMP-2 and are seeded with human umbilical vein endothelial cells and MSCs have shown increased osteogenesis and angiogenesis (Cui et al. 2016). Similarly, composite scaffolds that release VEGF and BMP-2 via different mechanisms have shown improved bone healing and vascularization when implanted into critical-size defects (Dawson et al. 2014; Curtin et al. 2015). However, the difficulties in the control of the in vivo release and maintenance of their

therapeutic levels, coupled with the potential risk of tumorigenesis and the high cost of growth factors, make this strategy a less attractive option for clinical translation.

To mimic the natural vasculature in tissues, engineered constructs should ideally contain mesovasculature and microvasculature in a hierarchical system, with post-implantation perfusion achieved by surgical anastomosis of the mesovasculature to the host blood vessels, or through rapid natural anastomosis between the construct and the host tissue. Table 8 gives the advantages and disadvantages of the most common methods used to create prevascularized tissue-engineered constructs with interconnected mesochannels and microchannels, which include electrospinning, molding, 3D printing, and laser degradation of scaffold biomaterials (Wang et al. 2019). Using a customized extrusion method printer, Kolesky and colleagues sequentially printed multiple bioinks that were composed of poly(dimethyl

Table 8 Advantages and disadvantages of the different methods used in vascular tissue engineering. (Adapted from Wang et al. 2019)

Method	Advantage	Disadvantage
Cell co-culture	Improved vasculogenesis and angiogenesis through cell interactions, no engineering apparatus needed	Unable to fabricate mesovasculature or thick constructs
Cell sheet stacking	Preservation of cell interaction and extracellular matrix	Long fabrication time, limited layers that can be stacked, cannot fabricate mesovasculature
In vivo scaffold culture	Rapid integration of mature vascular networks with host blood vessels naturally or surgically	Long culture time, multiple surgeries needed, which might cause donor tissue loss and complications
Decellularization	Preserved mesovasculature and microvasculature, and extracellular matrix	Difficult to precisely recellularize, incomplete decellularization can trigger immune response in vivo
Sacrificial molding	Fabrication of interconnected mesochannels with predetermined two-dimensional patterning	Complex manual processes, cannot fabricate three-dimensional vascular networks or microchannels
Sacrificial electrospinning	Fabrication of interconnected mesochannels and microchannels	Complex manual processes, random channel arrangement, uncontrollable channel size
Three-dimensional printing	Automated fabrication of mesochannels and microchannels with three-dimensional patterning using native tissue blood vessels, cell incorporation into process possible	Variable cell viability postbioprinting, cytotoxicity if stereolithography used, nozzle clogging common during extrusion bioprinting, construct dependent on biomaterial and scaffold design properties
Laser degradation	Automated fabrication of interconnected microchannels with a predesigned three-dimensional model, fast process, with high precision and resolution, relatively low damage to incorporated cells	Increased fabrication time for mesovasculature, limited thickness of fabricated tissue

siloxane), gelatin methacrylate, cell-based gelatin methacrylate, and 40% Pluronic F127. The 3D construct underwent ultraviolet processing to induce cross-linking in the gelatin methacrylate layers and was then cooled to 4 °C to liquefy the "sacrificial" Pluronic F127 layer, which was extracted using a vacuum. This left behind the interconnected microchannels that were dynamically seeded with human umbilical vein endothelial cells to form an "endothelialized" microvascular network (Kolesky et al. 2014). Kang and colleagues used 3D bioprinting to create scaffolds with highly interconnected microchannels that could support diffusion of nutrients and oxygen to enhance both vascularization and bone regeneration. The construct was fabricated using two bioinks: a cell-laden bioink that consisted of human amniotic-fluid-derived stem cells encapsulated in a hydrogel for tissue regeneration that was composed of gelatin, hyaluronic acid, fibrinogen, and glycerol, and a poly (caprolactone)-based ink to provide mechanical support. Following implantation in a rat calvarial bone defect, these bioprinted constructs regenerated vascularized bone tissue, with large blood vessels formed throughout the implants (Kang et al. 2016). However, such approaches have yet to show scalability. The universal issue faced by current vascularized tissue engineering methods is that endothelial cells do not sprout naturally or easily through synthetic extracellular matrices to allow remodeling or ready anastomosis with adjacent microvasculature and the host circulation. The structure of such generated capillary networks also tends to be poorly controlled and can regress easily. Until these problems are resolved, the creation of vascularized bone-tissue constructs will remain out of reach.

4 Clinical Applications of Bone-Marrow-Derived Mesenchymal Stromal Cells for Bone Regeneration

4.1 Preparation of Bone-Marrow-Derived Cell Therapies for Regenerative Medicine

Generally, cell therapies used for regenerative medicine can be administered as single-step or two-step procedures (Coelho et al. 2012; Čamernik et al. 2018; Marolt Presen et al. 2019). In single-step procedures, autologous tissue is harvested, processed (i.e., minimally manipulated), and transplanted during the surgical procedure. Bone marrow is a commonly used cell-therapy source for intraoperative procedures, apparently because it can be easily accessed in a rapid way, and because several tools are available for marrow harvesting (Coelho et al. 2012). Bone-marrow cell therapies come in different forms, depending on the method of bone-marrow aspirate processing. Most commonly, density-gradient centrifugation, automated tissue processing based on density gradients, immunomagnetic cell sorting, and selective cell retention are used to produce therapeutic preparations that contain mononuclear cells, mesenchymal and endothelial progenitor cells, and platelet-rich plasma (Coelho et al. 2012). It has been shown that the volume and technique of the bone-marrow aspiration affects the numbers of MSCs, with large volume aspirates

containing reduced numbers of colony-forming cells/osteoprogenitors, compared to smaller volume aspirates (Muschler et al. 1997; Hernigou et al. 2013). Currently, intraoperative stem-cell therapies still offer a more immediate potential for clinical applications, through integration of an autologous cell source with a well-established surgical intervention in a single procedure (Coelho et al. 2012).

Two-step procedures involve tissue harvesting during the first procedure, followed by in vitro cell culture in a GMP facility (see Sect. 4.2. for more details), with their administration as the second procedure. These cell therapies are thus considered to be more than minimally manipulated and are classified as advanced therapy medicinal products, which require regulatory clearance and approval (Ancans 2012; Sensebé et al. 2013). Both types of cell therapies can be administered via direct injection into the target tissue, by systemic injection, or by implantation in a surgical procedure in conjunction with a biocompatible scaffolding material (Kristjánsson and Honsawek 2014). Furthermore, different tissue-engineering approaches that involve bone-marrow MSCs are being investigated in preclinical and clinical studies (see Sects. 3.2.4. and 4.3) (Marolt Presen et al. 2019).

4.2 Culture and Characterization of Bone-Marrow-Derived Mesenchymal Stromal Cells for Clinical Use

Cultures of bone-marrow MSCs for clinical use have evolved from the procedures used in research studies (see Sect. 2.3.2; Table 2). The procedures have been adapted to exclude animal-derived culture components and to achieve appropriate scale-up of the MSCs for clinical therapies, in compliance with GMP regulations (Schallmoser et al. 2008; Agata et al. 2009; Chase et al. 2010; Fekete et al. 2012). The bone marrow of the patient is aspirated from the iliac crest in a standard manner and transported to the GMP laboratory for further processing. Schallmoser and colleagues developed a one-step protocol, where unprocessed heparinized bone marrow was directly diluted in culture medium, supplemented with pooled human platelet lysate (to replace fetal bovine serum), and cultured to obtain clinically relevant numbers of MSCs in primary culture (Schallmoser et al. 2008). Grisendi and colleagues compared density gradient media for bone-marrow MSC enrichment, to optimize the ex vivo expansion time (Grisendi et al. 2010). Chase and colleagues compared different serum-free media, supplemented with recombinant human PDGF-BB, bFGF, and TGFβ-1 (Chase et al. 2010). Wuchter and colleagues compared GMP-compliant medium supplemented with human platelet lysate with commercially available GMP-compliant media, for efficient and cost-effective expansion of bone-marrow MSCs (Wuchter et al. 2016), and Juhl and colleagues evaluated commercial human platelet lysates (Juhl et al. 2016). Fekete and colleagues compared five different GMP-compliant standardized protocols that were designed for safe, reliable, efficient, and economical isolation and expansion of MSCs, and they reported that they were all suitable to produce at least 100 million MSCs, which is commonly regarded as a single clinical dose (Fekete et al. 2012). The final MSC

products were equally effective according to the minimal criteria for MSCs. Robey and colleagues reported on a cell manufacturing procedure approved by the US Food and Drug Administration for ex vivo expansion of high-quality, biologically active human bone-marrow MSCs for use in bone regeneration. This included culture medium supplemented with fetal bovine serum as a cost-effective alternative, which required less testing for potential pathogens than the human-derived medium supplements (Robey et al. 2015).

These studies assessed the resulting MSCs and found that the cell properties were in agreement with the ISCT consensus criteria for plastic adherence, immunophenotype, and differentiation potential. As mentioned previously, the numbers and properties of MSCs vary between donors, anatomical site, and method of bone-marrow harvesting (Sect. 2.3.2.). In a review of MSC production according to GMP regulations, Sensebe and colleagues pointed out the lack of criteria to assess the normality or potency of donor MSCs, and therefore the difficulty to determine who will be a good MSC donor for therapies (Sensebé et al. 2013). This is particularly important in the case of allogeneic use, where large batches of MSCs are produced from one or a few donors. Thus far, most of the bone regeneration approaches using bone-marrow therapies have relied on the patient's own cells (Sect. 4.3.). Samsonraj and colleagues looked toward establishing the criteria for human MSC potency, for which they evaluated various in vitro and in vivo properties and showed that STRO-1 and PDGFRα were preferentially expressed on MSCs with high growth capacity (Samsonraj et al. 2015). Furthermore, these MSCs had the highest mean expression of TWIST-1 and DERMO-1 mRNA and produced higher amounts of mineralized tissue in ectopic bone formation assays, compared to the low-growth-capacity MSCs. However, they showed similar levels of key growth factors and cytokines involved in tissue regeneration (Samsonraj et al. 2015). Other groups investigated rapid methods to determine MSC potency. Murgia and colleagues reported on a combination of five globally relevant osteogenic signature genes, that is, the TGFβ-1 pathway interactors *ALPL*, *COL1A2*, *DCN*, *ELN*, and *RUNX2*, which could discriminate donor-specific bone-marrow MSCs (expanded under GMP-compliant conditions) according to their bone-forming potential in vivo (Murgia et al. 2016). Li and colleagues reported using fluorescent RNA probes to determine the relative intracellular levels of Sox9 and Runx2. This allowed prospective identification of osteogenic bone-marrow MSCs, as well as isolation within 1 week of relatively homogeneous cell populations with high osteogenic potential from the originally heterogeneous bone-marrow MSCs under osteogenic induction (Li et al. 2016).

In addition to potency evaluations and standard microbiological testing, MSC preparations must be examined before clinical use for occurrence and identification of any genetic alterations that might lead to MSC transformation into tumorigenic cells. Thus far, studies have shown that bone-marrow MSCs can be cultured over the long term with no risk of spontaneous malignant transformation (Bernardo et al. 2007), and since the first clinical trials, tumors have never been reported following the application of in vitro cultured MSCs (Sensebé et al. 2013).

4.3 Clinical Studies of Bone Regeneration with Bone-Marrow-Derived Cells

A number of preclinical studies in small and large animal models have evaluated bone-marrow-based cell therapies and BTE approaches to enhance bone regeneration. While some studies have shown no advantages of cultured cell transplantation (Stockmann et al. 2012), many have indicated positive effects on bone healing and have provided the basis for translation into the clinic (Bruder et al. 1998; Petite et al. 2000; Kawaguchi et al. 2004; Granero-Moltó et al. 2009; Nakamura et al. 2010; Caralla et al. 2013; Kandal et al. 2016). In 2005, Hernigou and colleagues reported on the treatment of fracture nonunions using fluoroscopy-controlled percutaneous injection of autologous mononuclear cells (MNCs) that they concentrated from bone-marrow aspirates by centrifugation (Hernigou et al. 2005). Successful bone union was obtained in 53 of 60 patients. These patients had significantly higher concentrations and total numbers of progenitor cells transplanted (evaluated using the CFU assay), as compared to the seven patients who did not achieve bone union. In addition, there was positive correlation between the total numbers and concentrations of transplanted CFUs and the volumes of the mineralized calluses at 4 months, and negative correlation between the times needed to obtain union and the concentrations of CFUs in the graft (Hernigou et al. 2005). Le Nail and colleagues analyzed a series of 43 patients with open tibial fractures with a risk of developing nonunions or who already presented with nonunions. Some of these patients received injections of concentrated bone-marrow progenitors, and from these a threshold number of transplanted progenitors required for successful healing was determined (Le Nail et al. 2014). Quarto and colleagues were the first to use cultured bone-marrow MSCs in conjunction with hydroxyapatite to treat large bone defects that resulted from traumatic fractures and unsuccessful lengthening in three patients (Quarto et al. 2001). They reported abundant callus formation along the implants, good integration of the implants with the host bones 2 months after surgery, and no adverse reactions, and all three of these patients recovered full limb function (Quarto et al. 2001).

Table 9 gives examples of other clinical studies that were initiated and are registered in the ClinicalTrials.gov database that have used bone-marrow-derived cell therapies or BTE approaches to enhance fracture healing, jaw-bone defect reconstruction, and osteonecrosis. These studies have used isolated bone-marrow MNCs and MSCs, culture-expanded MSCs, and MSCs predifferentiated into the osteogenic lineage. These cells were either directly injected into the bone defect or were delivered in combination with carrier biomaterials (Table 9). Overall, the data suggest that these therapies are safe and feasible and potentially have positive effects on bone regeneration. Liebergall and colleagues reported that a prophylactic injection of magnetically separated bone-marrow MSCs mixed with platelet-rich plasma and demineralized bone matrix (i.e., their study group of 12 patients) resulted in shorter times to reach bone union compared to the control group with conventional fracture treatment (12 patients) (Liebergall et al. 2013). Emadedin and colleagues reported that injections of cultured MSCs were safe for the treatment of fracture nonunions, with evidence of bone union in

Table 9 Clinical studies that have evaluated bone-marrow cell-based and tissue engineering therapies to enhance bone regeneration (as registered at ClinicalTrials.gov; July 2019). (Adapted from Marolt Presen et al. 2019)

System	Study identifier	Phase	Status	Condition	Cell type	Patients	Treatment groups	Main outcome	Reference
Fracture treatment – cultured MSCs	NCT03325504	III	Recruiting	Nonunion	Autologous bone-marrow MSCs (in vitro expanded)	~108	Low-dose stem-cell application with biomaterial; high-dose stem-cell application with biomaterial; control autologous bone graft		
	NCT01842477	I/II	February 2016	Delayed, union, nonunion	Autologous bone-marrow MSCs (cultured)	30	Application of stem cells with bone substitute	No severe adverse events; 26/28 treated patients radiologically healed at 1 year	Gómez-Barrena et al. 2019
	NCT02177565	NA	October 2011	Nonunion	Autologous bone-marrow MSCs (in vitro expanded)	35	Stem cell application with carrier, control as carrier alone		
	NCT01206179	I	March 2011	Nonunion	Autologous bone-marrow MSCs (in vitro expanded)	6	Stem-cell injection	Stem cell injections tolerated; evidence of union in 3/5 patients	Emadedin et al. 2017
	NCT00916981	I/II	June 2009	Nonunion, pseudo-arthrosis	Autologous bone-marrow-derived preosteoblastic cells	30	Preosteoblastic cell injection		

Fracture treatment	NCT01813188	II	December 2013	Pseudo-arthrosis	Autologous bone-marrow MNCs	5	Application of cells seeded on tricalcium phosphate		
– Isolated MNCs/MSCs	NCT01788059	II	November 2013	Nonunion	Autologous bone-marrow MSCs (Ficoll separated)	19	Stem-cell injection		
	NCT01581892	I/II	January 2013	Nonunion	Autologous bone-marrow MNCs (Ficoll separated)	7	Stem-cell injection		
	NCT00512434	NA	September 2013	Tibial fracture, open fracture	Autologous bone-marrow MNCs	85	Stem-cell injection and osteosynthesis, control as osteosynthesis only		
	NCT00250302	I/II	April 2011	Tibial fracture	Autologous bone-marrow MSCs (isolated)	24	Stem-cell implantation with autologous platelet-rich plasma/ demineralized bone carrier, control as no treatment	Shorter time to union in stem cell group (1.5 months) than control group (3 months)	Liebergall et al. 2013
Jaw bone regeneration	NCT02751125	I	Recruiting by invitation	Bone atrophy	Autologous bone-marrow MSCs (cultured)	13	Application of stem cells mixed with biphasic calcium phosphate	Bone formation sufficient for dental implant placement after 4–6 months	Gjerde et al. 2018
	NCT03070275	I/II	December 2017	Implant therapy	Autologous alveolar bone-	20	Application of stem cells with		

(continued)

Table 9 (continued)

System	Study identifier	Phase	Status	Condition	Cell type	Patients	Treatment groups	Main outcome	Reference
					marrow MSCs (in vitro expanded)		autologous fibrin glue in collagen scaffold		
	NCT02449005	I/II	December 2016	Chronic periodontitis	Autologous alveolar bone-marrow MSCs (in vitro expanded)	30	Application of stem cells with autologous fibrin glue in collagen scaffold, control as fibrin glue with collagen scaffold alone, control as no graft materials		
	NCT01389661	I/II	April 2016	Maxillary cyst, bone loss of substance	Autologous jaw bone-marrow MSCs (cultured, predifferentiated in osteogenic matrix)	11	Application of cells cultured in autologous plasma matrix	Increased cyst density by computed tomography; no adverse effects	Redondo et al. 2018
Osteonecrosis	NCT02448121	I/II	Active, not recruiting	Avascular necrosis of bone in patients with sickle cell disease	Autologous bone-marrow MNCs	~100	Stem-cell injection		

	NCT01605383	I/II	Active, not recruiting	Avascular necrosis of femoral head	Autologous bone-marrow MSCs (cultured)	~24	Application of cells with allogeneic bone, control as standard treatment only		
	NCT02065167	II	Active, not recruiting	Avascular necrosis of femoral head	Autologous bone-marrow MSCs (cultured)	26	Stem-cell injection		
	NCT01700920	II	December 2015	Osteonecrosis of femoral head	Autologous bone-marrow MSCs (cultured)	3	Stem-cell injection		
	NCT01198080	I	June 2013	Osteonecrosis of femoral head	Autologous CD133 bone-marrow cells	10	Stem-cell injection	Disease score improvement, reduced joint injuries, pain relief	Emadedin et al. 2019
	NCT01544712	NA	September 2010	Osteonecrosis of femoral head	Autologous bone-marrow aspirate concentrate	50	Bone-marrow concentrate injection, control as standard treatment only	Did not improve stage 3 osteonecrosis	Hauzeur et al. 2018
	NCT00821470	I	September 2008	Osteonecrosis of femoral head	Autologous bone-marrow aspirate	21	Bone-marrow injection, control as standard treatment only		

three of their five treated patients (Emadedin et al. 2017). Gomez-Barrena and colleagues reported that surgical delivery of culture-expanded bone-marrow MSCs combined with bioceramic granules for the treatment of delayed bone union and nonunion was feasible and safe, with 26 of 28 patients with radiological healing 1 year after treatment (Gómez-Barrena et al. 2019).

Redondo and colleagues treated nine patients with maxillary cysts using alveolar bone-marrow MSCs, which were osteogenically predifferentiated in vitro within autologous serum-derived scaffolds. They reported no adverse effects and increased density of the cyst interior, using computed tomography (Redondo et al. 2018). Similarly, Gjerde and colleagues reported their treatment of 13 patients who had severely atrophied mandibular bone, where they used bone-marrow MSCs mixed with biphasic calcium phosphate. They found no adverse events, and there was sufficient bone regeneration for implant placement after 4–6 months (Gjerde et al. 2018).

Therapies that involve autologous bone-marrow MNCs and MSCs have also been evaluated for the treatment of femoral head osteonecrosis (Table 9) (Hernigou and Beaujean 2002; Hernigou et al. 2018). Hernigou and colleagues reported that supplementation of the core decompensation procedure with concentrated bone-marrow MNC injections was effective for the treatment of patients with early stage disease, which resulted in fewer hip replacements. Better outcomes were shown for the patients who had higher numbers of progenitors transplanted (Hernigou and Beaujean 2002). A follow-up study after 20–30 years found that core decompression with bone-marrow-cell injections improved the outcome of the disease (again, with less hip replacements), as compared with core decompression alone in the same patient group (Hernigou et al. 2018). More recently, Emadedin and colleagues reported using injections of magnetically separated $CD133^{+}$ bone-marrow progenitors in nine patients with femoral head osteonecrosis. This treatment resulted in improved disease scores and fewer joint injuries, and provided clinically relevant pain relief (Emadedin et al. 2019). In contrast, Hauzeur and colleagues reported that implantation of concentrated bone-marrow MNCs after core decompression did not produce any improvements in the progression of stage 3 non-traumatic osteonecrosis of the femoral head (Hauzeur et al. 2018). Finally, ongoing registered studies are seeking to determine the effects of transplanted cells versus carrier materials alone, and the effects of different stem-cell doses on bone regeneration (Table 9).

5 Challenges and Future Directions

The translation of MSC-based cell therapies and tissue engineering approaches to the clinic has been slow, and to date, there are no MSC-based therapeutic products for bone regeneration that have been accepted as appropriate standard of care. In vivo studies of bone regeneration have shown limited survival and engraftment of transplanted bone-marrow MSCs in bone defects. Nevertheless, the existence of a renewable population of bone stem cells in bone marrow means that there remain exciting possibilities for cell-based bone regeneration and repair, given the relative accessibility of autologous tissue.

Challenges remain, however, including our limited understanding of skeletal stem-cell fate and immuno-phenotypes, and the need for easier selection, enrichment, expansion, and scale-up of these cells that, to date, have precluded widespread clinical application. In contrast to the initially postulated direct differentiation of MSCs into bone cells to form new tissue after transplantation, in vitro and in vivo studies have also indicated a paracrine mechanism on endogenous cell populations. Increasing knowledge around MSC trophic and immunomodulatory activities has steered current research toward MSCs as the source of cell-free, "off-the-shelf" therapeutic preparations (e.g., secretome, extracellular vesicles) to enhance various stages of bone regeneration (reviewed by Marolt Presen et al. (2019)).

In the context of bone regeneration, the use of therapeutic MSC-derived secretome-containing mixtures of growth/signaling factors and various extracellular vesicles might facilitate the native processes of bone healing, with the various multiple signaling factors acting in synergy at different stages of bone regeneration (Fig. 3). In addition, secretome-based (cell-free) therapeutic approaches have several advantages over current cell-based and BTE therapies. The apparent absence of replicating (allogeneic) cells in secretome fractions offers, potentially: (i) improved patient safety profile; (ii) improved quality control and quality assurance, given the low metabolic profile; and (iii) simplicity of storage, which provides for less complex logistics for clinical use of this potentially off-the-shelf therapeutic substance (Marolt Presen et al. 2019). Interestingly, not only bone-marrow MSCs, but also alternative cell types (discussed in Sect. 3.2.4.) are under investigation for cell-based and cell-free bone regenerative therapies. Comparative studies using standardized MSC-based therapeutic preparations will advance our understanding of the appropriate therapeutic approaches for musculoskeletal applications, which has tremendous implications for an aging demographic.

Bone-marrow-derived MSCs have clinical indications far beyond bone regeneration. To date, bone-marrow-derived MSCs have been included in clinical trials for more than 500 conditions, most of which relate to musculoskeletal disorders. However, numerous clinical trials are investigating the efficacy of bone-marrow-derived MSCs for conditions such as immune system diseases (e.g., graft-versus-host disease, multiple sclerosis, rheumatoid arthritis), heart diseases (e.g., myocardial infarction, cardiomyopathy, heart failure), gastrointestinal disease (e.g., liver failure, Crohn's disease), central nervous system diseases (e.g., stroke, spinal cord injury), and wounds, and many others (search: ClinicalTrials.gov database, using the term "bone-marrow mesenchymal stem cells"; February 2020). Studies and review papers that provide evidence for the application of bone-marrow-derived MSCs for some of these clinical conditions are given in Table 10.

6 Conclusions

Since the first discovery of bone-marrow stromal cells some 50 years ago, growing clinical interest and extensive research efforts have been focused on an understanding of the identity and potential of these cells for regenerative medicine. Using transgenic animal models for lineage tracing, various subpopulations of MSCs with roles in bone

Table 10 Clinical indications beyond bone regeneration for the use of bone-marrow-derived mesenchymal stromal cells

Clinical indication	References
Osteoarthritis	Harrell et al. 2019; Chahal et al. 2019
Graft-versus-host disease	Dunavin et al. 2017; Elgaz et al. 2019; Zhao et al. 2019
Inflammatory bowel disease	Kang et al. 2018; Volarevic et al. 2019
Myocardial infarction	Miao et al. 2017
Multiple sclerosis	Uccelli et al. 2019

development and regeneration have been identified. To date numerous preclinical investigations have evaluated the potential of bone-marrow MSCs for cell therapies and BTE applications, both alone and in conjunction with various biomaterials and signaling molecules. The positive outcomes of most of these studies have paved the way for transition into clinical studies, which have to date been typically for treatment of bone fractures, jaw-bone defect regeneration, and osteonecrosis. However, only a limited number of clinical trials have published their research findings at present (see Table 9), and the optimal therapeutic strategies for specific clinical indications remain under evaluation. Vascularization remains a key unresolved challenge to advance the field, given the critical role of vascularization in the support of the survival of transplanted cells and BTE grafts, and in the provision of nourishment for the regenerating bone tissue. However, the recent advances that have involved the identification of human skeletal stem cells, the advances in the standardization of the characterization of appropriate therapeutic cells and their derivatives, and the recent methodological advances (e.g., additive manufacturing) now augur well for the application of bone-marrow MSCs for bone regeneration and for the final realization of the promise of marrow-derived stromal stem cells.

Acknowledgments This work was supported by the European Union Horizon 2020 Research and Innovation Programme under Marie Sklodowska-Curie grant agreement No. 657716 (to DMP), and by the Austrian Research Promotion Agency (FFG) grant No. 867803 (SINEM). This work was also supported by funding from the Slovenian Research Agency (J3-1749; to JZ). Research support to RO from the Biotechnology and Biological Sciences Research Council (BBSRC LO21071/1, BB/L00609X/1, BB/P017711/1), the UK Regenerative Medicine Platform Acellular/Smart Materials – 3D Architecture (MR/R015651/1), Rosetrees Trust, Wessex Medical Research, and the University of Southampton is gratefully acknowledged, along with the many useful discussions with past and current members of the Bone and Joint Research Group in Southampton, UK. The Figures were created using Mind the Graph. The authors thank Dr. Chris Berrie for scientific English editing of the manuscript.

References

Agata H, Watanabe N, Ishii Y, Kubo N, Ohshima S, Yamazaki M, Tojo A, Kagami H (2009) Feasibility and efficacy of bone tissue engineering using human bone marrow stromal cells cultivated in serum-free conditions. Biochem Biophys Res Commun. https://doi.org/10.1016/j.bbrc.2009.03.023

Ancans J (2012) Cell therapy medicinal product regulatory framework in Europe and its application for MSC-based therapy development. Front Immunol 3:253

Beloti MM, Sicchieri LG, De Oliveira PT, Rosa AL (2012) The influence of osteoblast differentiation stage on bone formation in autogenously implanted cell-based poly(lactide-co-glycolide) and calcium phosphate constructs. Tissue Eng Part A. https://doi.org/10.1089/ten.tea.2011.0405

Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F (2007) Human bone marrow-derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-06-4690

Bhumiratana S, Eton RE, Oungoulian SR, Wan LQ, Ateshian GA, Vunjak-Novakovic G (2014) Large, stratified, and mechanically functional human cartilage grown in vitro by mesenchymal condensation. Proc Natl Acad Sci USA 111:6940–6945. https://doi.org/10.1073/pnas.1324050111

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. https://doi.org/10.1016/S0014-4827(03)00138-1

Boskey AL (2013) Bone composition: relationship to bone fragility and antiosteoporotic drug effects. Bonekey Rep. https://doi.org/10.1038/bonekey.2013.181

Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM (2013) Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 15:641–648. https://doi.org/10.1016/j.jcyt.2013.02.006

Bruder SP, Kraus KH, Goldberg VM, Kadiyala S (1998) The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Jt Surg A. https://doi.org/10.2106/00004623-199807000-00007

Čamernik K, Barlič A, Drobnič M, Marc J, Jeras M, Zupan J (2018) Mesenchymal stem cells in the musculoskeletal system: from animal models to human tissue regeneration? Stem Cell Rev Rep 14:346–369. https://doi.org/10.1007/s12015-018-9800-6

Campbell TM, Churchman SM, Gomez A, Mcgonagle D, Conaghan PG, Ponchel F, Jones E (2016) Mesenchymal stem cell alterations in bone marrow lesions in patients with hip osteoarthritis. Arthritis Rheumatol 68:1648–1659. https://doi.org/10.1002/art.39622

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650. https://doi.org/10.1002/jor.1100090504

Caplan AI (2017) Mesenchymal stem cells: time to change the name! Stem Cells Transl Med 6:1445–1451. https://doi.org/10.1002/sctm.17-0051

Caralla T, Joshi P, Fleury S, Luangphakdy V, Shinohara K, Pan H, Boehm C, Vasanji A, Hefferan TE, Walker E, Yaszemski M, Hascall V, Zborowski M, Muschler GF (2013) In vivo transplantation of autogenous marrow-derived cells following rapid intraoperative magnetic separation based on hyaluronan to augment bone regeneration. Tissue Eng Part A. https://doi.org/10.1089/ten.tea.2011.0622

Cattaneo P, Sun Y, Moore-morris T, Dalton ND, Rockenstein E, Masliah E, Peterson KL, Stallcup B, Chen J, Evans SM, Jolla L, Hospital E, Jolla L, Jolla L, Program CI, Burnham S, Jolla L, Jolla L (2018) Pericytes of multiple organs. Cell Stem Cell 20:345–359. https://doi.org/10.1016/j.stem.2016.12.006.Pericytes

Cavallo C, Cuomo C, Fantini S, Ricci F, Tazzari PL, Lucarelli E, Donati D, Facchini A, Lisignoli G, Fornasari PM, Grigolo B, Moroni L (2011) Comparison of alternative mesenchymal stem cell sources for cell banking and musculoskeletal advanced therapies. J Cell Biochem 112:1418–1430. https://doi.org/10.1002/jcb.23058

Chahal J, Gómez-Aristizábal A, Shestopaloff K, Bhatt S, Chaboureau A, Fazio A, Chisholm J, Weston A, Chiovitti J, Keating A, Kapoor M, Ogilvie-Harris DJ, Syed KA, Gandhi R, Mahomed NN, Marshall KW, Sussman MS, Naraghi AM, Viswanathan S (2019) Bone marrow mesenchymal stromal cell treatment in patients with osteoarthritis results in overall improvement in pain and symptoms and reduces synovial inflammation. Stem Cells Transl Med 8:746–757. https://doi.org/10.1002/sctm.18-0183

Chan CKF, Seo EY, Chen JY, Lo D, McArdle A, Sinha R, Tevlin R, Seita J, Vincent-Tompkins J, Wearda T, Lu WJ, Senarath-Yapa K, Chung MT, Marecic O, Tran M, Yan KS, Upton R, Walmsley GG, Lee AS, Sahoo D, Kuo CJ, Weissman IL, Longaker MT (2015) Identification and specification of the mouse skeletal stem cell. Cell 160:285–298. https://doi.org/10.1016/j.cell.2014.12.002

Chan CKF, Gulati GS, Sinha R, Tompkins JV, Lopez M, Carter AC, Ransom RC, Reinisch A, Wearda T, Murphy M, Brewer RE, Koepke LS, Marecic O, Manjunath A, Seo EY, Leavitt T, Lu W-J, Nguyen A, Conley SD, Salhotra A, Ambrosi TH, Borrelli MR, Siebel T, Chan K, Schallmoser K, Seita J, Sahoo D, Goodnough H, Bishop J, Gardner M, Majeti R, Wan DC, Goodman S, Weissman IL, Chang HY, Longaker MT (2018) Identification of the human skeletal stem cell. Cell 175:43–56.e21. https://doi.org/10.1016/j.cell.2018.07.029

Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC (2010) A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem Cell Res Ther. https://doi.org/10.1186/scrt8

Chen L, Tredget EE, Wu PYG, Wu Y, Wu Y (2008) Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 3. https://doi.org/10.1371/journal.pone.0001886

Cheng H, Qiu L, Ma J, Zhang H, Cheng M, Li W, Zhao X, Liu K (2011) Replicative senescence of human bone marrow and umbilical cord derived mesenchymal stem cells and their differentiation to adipocytes and osteoblasts. Mol Biol Rep. https://doi.org/10.1007/s11033-010-0665-2

Coelho MB, Cabral JMS, Karp JM (2012) Intraoperative stem cell therapy. Annu Rev Biomed Eng 14:325–349. https://doi.org/10.1146/annurev-bioeng-071811-150041

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Péault B (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313. https://doi.org/10.1016/j.stem.2008.07.003

Csobonyeiova M, Polak S, Zamborsky R, Danisovic L (2017) iPS cell technologies and their prospect for bone regeneration and disease modeling: a mini review. J Adv Res 8:321

Cui H, Zhu W, Holmes B, Zhang LG (2016) Biologically inspired smart release system based on 3D bioprinted perfused scaffold for vascularized tissue regeneration. Adv Sci. https://doi.org/10.1002/advs.201600058

Curtin CM, Tierney EG, Mcsorley K, Cryan SA, Duffy GP, O'Brien FJ (2015) Combinatorial gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold. Adv Healthc Mater. https://doi.org/10.1002/adhm.201400397

Dalby MJ, Gadegaard N, Oreffo ROC (2014) Harnessing nanotopography and integrin-matrix interactions to influence stem cell fate. Nat Mater. https://doi.org/10.1038/nmat3980

Dang M, Saunders L, Niu X, Fan Y, Ma PX (2018) Biomimetic delivery of signals for bone tissue engineering. Bone Res 6:25. https://doi.org/10.1038/s41413-018-0025-8. eCollection 2018.

Dawson JI, Kanczler J, Tare R, Kassem M, Oreffo ROC (2014) Concise review: bridging the gap: bone regeneration using skeletal stem cell-based strategies-where are we now? Stem Cells 32:35

de Peppo GM, Svensson S, Lennerås M, Synnergren J, Stenberg J, Strehl R, Hyllner J, Thomsen P, Karlsson C (2010) Human embryonic mesodermal progenitors highly resemble human mesenchymal stem cells and display high potential for tissue engineering applications. Tissue Eng Part A 16:2161–2182. https://doi.org/10.1089/ten.TEA.2009.0629

Di Maggio N, Mehrkens A, Papadimitropoulos A, Schaeren S, Heberer M, Banfi A, Martin I (2012) Fibroblast growth factor-2 maintains a niche-dependent population of self-renewing highly potent non-adherent mesenchymal progenitors through FGFR2c. Stem Cells 30:1455–1464. https://doi.org/10.1002/stem.1106

Ding L, Saunders TL, Enikolopov G, Morrison SJ (2012) Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 481:457–462. https://doi.org/10.1038/nature10783

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal

cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317. https://doi.org/10.1080/14653240600855905
Dunavin N, Dias A, Li M, McGuirk J (2017) Mesenchymal stromal cells: what is the mechanism in acute graft-versus-host disease? Biomedicine 5:39
Einhorn TA, Gerstenfeld LC (2015) Fracture healing: mechanisms and interventions. Nat Rev Rheumatol 11:45–54
Elgaz S, Kuçi Z, Kuçi S, Bönig H, Bader P (2019) Clinical use of mesenchymal stromal cells in the treatment of acute graft-versus-host disease. Transfus Med Hemother 46:27–34. https://doi.org/10.1159/000496809
Emadedin M, Labibzadeh N, Fazeli R, Mohseni F, Hosseini SE, Moghadasali R, Mardpour S, Azimian V, Goodarzi A, Liastani MG, Bafghi AM, Eslaminejad MB, Aghdami N (2017) Percutaneous autologous bone marrow-derived mesenchymal stromal cell implantation is safe for reconstruction of human lower limb long bone atrophic nonunion. Cell J. https://doi.org/10.22074/cellj.2016.4866
Emadedin M, Karimi S, Karimi A, Labibzadeh N, Niknejadi M, Baharvand HAN (2019) Autologous bone marrow–derived CD133 cells with core decompression as a novel treatment method for femoral head osteonecrosis: a pilot study. Cytotherapy. https://doi.org/10.1016/j.jcyt.2018.10.005
Fedorovich NE, Haverslag RT, Dhert WJA, Alblas J (2010) The role of endothelial progenitor cells in prevascularized bone tissue engineering: development of heterogeneous constructs. Tissue Eng Part A. https://doi.org/10.1089/ten.tea.2009.0603
Fedorovich NE, Alblas J, Hennink WE, Öner FC, Dhert WJA (2011) Organ printing: the future of bone regeneration? Trends Biotechnol 29:601–606
Feil S, Valtcheva N, Feil R (2009) Inducible cre mice. Methods Mol Biol. https://doi.org/10.1007/978-1-59745-471-1_18
Fekete N, Rojewski MT, Fürst D, Kreja L, Ignatius A, Dausend J, Schrezenmeier H (2012) GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. PLoS One. https://doi.org/10.1371/journal.pone.0043255
Fernandez-Yague MA, Abbah SA, McNamara L, Zeugolis DI, Pandit A, Biggs MJ (2015) Biomimetic approaches in bone tissue engineering: integrating biological and physicomechanical strategies. Adv Drug Deliv Rev
Frank O, Heim M, Jakob M, Barbero A, Schäfer D, Bendik I, Dick W, Heberer M, Martin I (2002) Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem 85:737–746. https://doi.org/10.1002/jcb.10174
Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Prolif 3:393–403. https://doi.org/10.1111/j.1365-2184.1970.tb00347.x
Frohlich M, Grayson W, Wan L, Marolt D, Drobnic M, Vunjak-Novakovic G (2008) Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Curr Stem Cell Res Ther. https://doi.org/10.2174/157488808786733962
Gao F, Chiu SM, Motan DAL, Zhang Z, Chen L, Ji HL, Tse HF, Fu QL, Lian Q (2016) Mesenchymal stem cells and immunomodulation: current status and future prospects. Cell Death Dis 7. https://doi.org/10.1038/cddis.2015.327
García Quiroz F, Posada Estefan O, Gallego Pérez D, Higuita Castro N, Sarassa Velásquez C, Hansford D, Agudelo Florez P, López Rojas L (2008) Isolation of human bone marrow mesenchymal stem cells and evaluation of their osteogenic potential. Rev Ing Bioméd. https://doi.org/10.24050/19099762.n3.2008.43
Gibbs DMR, Vaezi M, Yang S, Oreffo ROC (2014) Hope versus hype: what can additive manufacturing realistically offer trauma and orthopedic surgery? Regen Med 9:535
Gibon E, Lu L, Goodman SB (2016) Aging, inflammation, stem cells, and bone healing. Stem Cell Res Ther 7:44
Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100:1249–1260

Gjerde C, Mustafa K, Hellem S, Rojewski M, Gjengedal H, Yassin MA, Feng X, Skaale S, Berge T, Rosen A, Shi XQ, Ahmed AB, Gjertsen BT, Schrezenmeier H, Layrolle P (2018) Cell therapy induced regeneration of severely atrophied mandibular bone in a clinical trial. Stem Cell Res Ther. https://doi.org/10.1186/s13287-018-0951-9

Glenn JD (2014) Mesenchymal stem cells: emerging mechanisms of immunomodulation and therapy. World J Stem Cells 6:526. https://doi.org/10.4252/wjsc.v6.i5.526

Goggin PM, Zygalakis KC, Oreffo ROC, Schneider P (2016) High-resolution 3D imaging of osteocytes and computational modelling in mechanobiology: insights on bone development, ageing, health and disease. Eur Cells Mater. https://doi.org/10.22203/eCM.v031a18

Gómez-Barrena E, Rosset P, Gebhard F, Hernigou P, Baldini N, Rouard H, Sensebé L, Gonzalo-Daganzo RM, Giordano R, Padilla-Eguiluz N, García-Rey E, Cordero-Ampuero J, Rubio-Suárez JC, Stanovici J, Ehrnthaller C, Huber-Lang M, Flouzat-Lachaniette CH, Chevallier N, Donati DM, Ciapetti G, Fleury S, Fernandez MN, Cabrera JR, Avendaño-Solá C, Montemurro T, Panaitescu C, Veronesi E, Rojewski MT, Lotfi R, Dominici M, Schrezenmeier H, Layrolle P (2019) Feasibility and safety of treating non-unions in tibia, femur and humerus with autologous, expanded, bone marrow-derived mesenchymal stromal cells associated with biphasic calcium phosphate biomaterials in a multicentric, non-comparative trial. Biomaterials. https://doi.org/10.1016/j.biomaterials.2018.03.033

Gothard D, Smith EL, Kanczler JM, Rashidi H, Qutachi O, Henstock J, Rotherham M, El Haj A, Shakesheff KM, Oreffo ROC (2014) Tissue engineered bone using select growth factors: a comprehensive review of animal studies and clinical translation studies in man. Eur Cells Mater. https://doi.org/10.22203/eCM.v028a13

Granero-Moltó F, Weis JA, Miga MI, Landis B, Myers TJ, O'Rear L, Longobardi L, Jansen ED, Mortlock DP, Spagnoli A (2009) Regenerative effects of transplanted mesenchymal stem cells in fracture healing. Stem Cells. https://doi.org/10.1002/stem.103

Grayson WL, Fröhlich M, Yeager K, Bhumiratana S, Chan ME, Cannizzaro C, Wan LQ, Liu XS, Guo XE, Vunjak-Novakovic G (2010) Engineering anatomically shaped human bone grafts. Proc Natl Acad Sci USA. https://doi.org/10.1073/pnas.0905439106

Grayson WL, Marolt D, Bhumiratana S, Fröhlich M, Guo XE, Vunjak-Novakovic G (2011) Optimizing the medium perfusion rate in bone tissue engineering bioreactors. Biotechnol Bioeng. https://doi.org/10.1002/bit.23024

Grcevic D, Pejda S, Matthews BG, Repic D, Wang L, Li H, Kronenberg MS, Jiang X, Maye P, Adams DJ, Rowe DW, Aguila HL, Kalajzic I (2012) In vivo fate mapping identifies mesenchymal progenitor cells. Stem Cells 30:187–196. https://doi.org/10.1002/stem.780

Grisendi G, Annerén C, Cafarelli L, Sternieri R, Veronesi E, Cervo GL, Luminari S, Maur M, Frassoldati A, Palazzi G, Otsuru S, Bambi F, Paolucci P, Pierfranco C, Horwitz E, Dominici M (2010) GMP-manufactured density gradient media for optimized mesenchymal stromal/stem cell isolation and expansion. Cytotherapy 12:466–477. https://doi.org/10.3109/14653241003649510

Guo Z, Li H, Li X, Yu X, Wang H, Tang P, Mao N (2006) In vitro characteristics and in vivo immunosuppressive activity of compact bone-derived murine mesenchymal progenitor cells. Stem Cells 24:992–1000. https://doi.org/10.1634/stemcells.2005-0224

Harrell CR, Markovic BS, Fellabaum C, Arsenijevic A, Volarevic V (2019) Mesenchymal stem cell-based therapy of osteoarthritis: current knowledge and future perspectives. Biomed Pharmacother 109:2318

Haumer A, Bourgine PE, Occhetta P, Born G, Tasso R, Martin I (2018) Delivery of cellular factors to regulate bone healing. Adv Drug Deliv Rev 129:285

Hauzeur JP, De Maertelaer V, Baudoux E, Malaise M, Beguin Y, Gangji V (2018) Inefficacy of autologous bone marrow concentrate in stage three osteonecrosis: a randomized controlled double-blind trial. Int Orthop. https://doi.org/10.1007/s00264-017-3650-8

Hernigou P, Beaujean F (2002) Treatment of osteonecrosis with autologous bone marrow grafting. In: Clinical Orthopaedics and related research. Lippincott Williams & Wilkins, Hagerstown

Hernigou P, Poignard A, Beaujean F, Rouard H (2005) Percutaneous autologous bone-marrow grafting for nonunions: influence of the number and concentration of progenitor cells. J Bone Jt Surg A. https://doi.org/10.2106/JBJS.D.02215

Hernigou P, Homma Y, Flouzat Lachaniette CH, Poignard A, Allain J, Chevallier N, Rouard H (2013) Benefits of small volume and small syringe for bone marrow aspirations of mesenchymal stem cells. Int Orthop 37:2279–2287. https://doi.org/10.1007/s00264-013-2017-z

Hernigou P, Dubory A, Homma Y, Guissou I, Flouzat Lachaniette CH, Chevallier N, Rouard H (2018) Cell therapy versus simultaneous contralateral decompression in symptomatic corticosteroid osteonecrosis: a thirty year follow-up prospective randomized study of one hundred and twenty five adult patients. Int Orthop. https://doi.org/10.1007/s00264-018-3941-8

Herrmann M, Hildebrand M, Menzel U, Fahy N, Alini M, Lang S, Benneker L, Verrier S, Stoddart MJ, Bara JJ (2019) Phenotypic characterization of bone marrow mononuclear cells and derived stromal cell populations from human iliac crest, vertebral body and femoral head. Int J Mol Sci. https://doi.org/10.3390/ijms20143454

Hofer HR, Tuan RS (2016) Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies. Stem Cell Res Ther 7:1–14. https://doi.org/10.1186/s13287-016-0394-0

Hofmann S, Hagenmüller H, Koch AM, Müller R, Vunjak-Novakovic G, Kaplan DL, Merkle HP, Meinel L (2007) Control of in vitro tissue-engineered bone-like structures using human mesenchymal stem cells and porous silk scaffolds. Biomaterials. https://doi.org/10.1016/j.biomaterials.2006.10.019

Ho-Shui-Ling A, Bolander J, Rustom LE, Johnson AW, Luyten FP, Picart C (2018) Bone regeneration strategies: engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives. Biomaterials 180:143

Houlihan DD, Mabuchi Y, Morikawa S, Niibe K, Araki D, Suzuki S, Okano H, Matsuzaki Y (2012) Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR-α. Nat Protoc 7:2103–2111. https://doi.org/10.1038/nprot.2012.125

Hung BP, Hutton DL, Kozielski KL, Bishop CJ, Naved B, Green JJ, Caplan AI, Gimble JM, Dorafshar AH, Grayson WL (2015) Platelet-derived growth factor BB enhances Osteogenesis of adipose-derived but not bone marrow-derived mesenchymal stromal/stem cells. Stem Cells 33:2773–2784. https://doi.org/10.1002/stem.2060

Ikebe C, Suzuki K (2014) Mesenchymal stem cells for regenerative therapy: optimization of cell preparation protocols. Biomed Res Int 2014:1–11. https://doi.org/10.1155/2014/951512

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312. https://doi.org/10.1002/(SICI)1097-4644(199702)64:2<295::AID-JCB12>3.0.CO;2-I

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272. https://doi.org/10.1006/excr.1997.3858

Jones E, McGonagle D (2008) Human bone marrow mesenchymal stem cells in vivo. Rheumatology 47:126–131. https://doi.org/10.1093/rheumatology/kem206

Jones E, English A, Churchman SM, Kouroupis D, Boxall SA, Kinsey S, Giannoudis PG, Emery P, McGonagle D (2010) Large-scale extraction and characterization of CD271+ multipotential stromal cells from trabecular bone in health and osteoarthritis: implications for bone regeneration strategies based on uncultured or minimally cultured multipotential stromal cells. Arthritis Rheum 62:1944–1954. https://doi.org/10.1002/art.27451

Juhl M, Tratwal J, Follin B, Søndergaard RH, Kirchhoff M, Ekblond A, Kastrup J, Haack-Sørensen M (2016) Comparison of clinical grade human platelet lysates for cultivation of mesenchymal stromal cells from bone marrow and adipose tissue. Scand J Clin Lab Invest. https://doi.org/10.3109/00365513.2015.1099723

Kanczler JM, Oreffo ROC (2008) Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater 15:100

Kandal S, Özmen S, Uygur S, Yagci M, Kayhan H, Elmas C, Araç M, Çelebi C (2016) Effects of rat bone marrow-derived mesenchymal stem cells and demineralized bone matrix on cranial bone healing. Ann Plast Surg 77:249–254. https://doi.org/10.1097/SAP.0000000000000274

Kang HW, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A (2016) A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. Nat Biotechnol. https://doi.org/10.1038/nbt.3413

Kang J, Zhang L, Luo X, Ma X, Wang G, Yang Y, Yan Y, Qian H, Zhang X, Xu W, Mao F (2018) Systematic exposition of mesenchymal stem cell for inflammatory bowel disease and its associated colorectal cancer. Biomed Res Int 2018:1

Kawaguchi H, Hirachi A, Hasegawa N, Iwata T, Hamaguchi H, Shiba H, Takata T, Kato Y, Kurihara H (2004) Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. J Periodontol. https://doi.org/10.1902/jop.2004.75.9.1281

Keriquel V, Oliveira H, Rémy M, Ziane S, Delmond S, Rousseau B, Rey S, Catros S, Amédée J, Guillemot F, Fricain JC (2017) In situ printing of mesenchymal stromal cells, by laser-assisted bioprinting, for in vivo bone regeneration applications. Sci Rep. https://doi.org/10.1038/s41598-017-01914-x

Kesireddy V, Kasper FK (2016) Approaches for building bioactive elements into synthetic scaffolds for bone tissue engineering. J Mater Chem B 4:6773

Kim BS, Shkembi F, Lee J (2017) In vitro and in vivo evaluation of commercially available fibrin gel as a carrier of alendronate for bone tissue engineering. Biomed Res Int. https://doi.org/10.1155/2017/6434169

Kim H, Kim M, Im S-K, Fang S (2018) Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. Lab Anim Res 34:147–159. https://doi.org/10.5625/lar.2018.34.4.147

Ko E, Alberti K, Lee JS, Yang K, Jin Y, Shin J, Yang HS, Xu Q, Cho S-W (2016) Nanostructured tendon-derived scaffolds for enhanced bone regeneration by human adipose-derived stem cells. ACS Appl Mater Interfaces 8:22819–22829. https://doi.org/10.1021/acsami.6b05358

Kobolak J, Dinnyes A, Memic A, Khademhosseini A, Mobasheri A (2016) Mesenchymal stem cells: identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche. Methods 99:62–68

Kolesky DB, Truby RL, Gladman AS, Busbee TA, Homan KA, Lewis JA (2014) 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. Adv Mater. https://doi.org/10.1002/adma.201305506

Koons GL, Mikos AG (2019) Progress in three-dimensional printing with growth factors. J Control Release. https://doi.org/10.1016/j.jconrel.2018.12.035

Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F (2006) Role for interferon-γ in the Immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 24:386–398. https://doi.org/10.1634/stemcells.2005-0008

Kristjánsson B, Honsawek S (2014) Current perspectives in mesenchymal stem cell therapies for osteoarthritis. Stem Cells Int 2014:1

Kumar R, Godavarthy PS, Krause DS (2018) The bone marrow microenvironment in health and disease at a glance. J Cell Sci 131:jcs201707. https://doi.org/10.1242/jcs.201707

Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, Mizoguchi T, Wei Q, Lucas D, Ito K, Mar JC, Bergman A, Frenette PS (2013) Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 502:637–643. https://doi.org/10.1038/nature12612

Lai Y, Sun Y, Skinner CM, Son EL, Lu Z, Tuan RS, Jilka RL, Ling J, Chen XD (2010) Reconstitution of marrow-derived extracellular matrix ex vivo: a robust culture system for expanding large-scale highly functional human mesenchymal stem cells. Stem Cells Dev 19:1095–1107. https://doi.org/10.1089/scd.2009.0217

Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringdén O (2003) Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and Mitogenic responses independently of the

major histocompatibility complex. Scand J Immunol 57:11–20. https://doi.org/10.1046/j.1365-3083.2003.01176.x

Le Nail LR, Stanovici J, Fournier J, Splingard M, Domenech J, Rosset P (2014) Percutaneous grafting with bone marrow autologous concentrate for open tibia fractures: analysis of forty three cases and literature review. Int Orthop. https://doi.org/10.1007/s00264-014-2342-x

Lee H-S, Huang G-T, Chiang H, Chiou L-L, Chen M-H, Hsieh C-H, Jiang C-C (2003) Multipotential mesenchymal stem cells from femoral bone marrow near the site of osteonecrosis. Stem Cells 21:190–199. https://doi.org/10.1634/stemcells.21-2-190

Leijten J, Chai YC, Papantoniou I, Geris L, Schrooten J, Luyten FP (2015) Cell based advanced therapeutic medicinal products for bone repair: keep it simple? Adv Drug Deliv Rev 84:30

Léotot J, Lebouvier A, Hernigou P, Bierling P, Rouard H, Chevallier N (2015) Bone-forming capacity and biodistribution of bone marrow-derived stromal cells directly loaded into scaffolds: a novel and easy approach for clinical application of bone regeneration. Cell Transplant. https://doi.org/10.3727/096368914X685276

Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, Tuan RS (2005) A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 26:599–609. https://doi.org/10.1016/j.biomaterials.2004.03.005

Li B, Menzel U, Loebel C, Schmal H, Alini M, Stoddart MJ (2016) Monitoring live human mesenchymal stromal cell differentiation and subsequent selection using fluorescent RNA-based probes. Sci Rep. https://doi.org/10.1038/srep26014

Liebergall M, Schroeder J, Mosheiff R, Gazit Z, Yoram Z, Rasooly L, Daskal A, Khoury A, Weil Y, Beyth S (2013) Stem cell-based therapy for prevention of delayed fracture union: a randomized and prospective preliminary study. Mol Ther. https://doi.org/10.1038/mt.2013.109

Liu Y, Teoh SH, Chong MSK, Yeow CH, Kamm RD, Choolani M, Chan JKY (2013) Contrasting effects of vasculogenic induction upon biaxial bioreactor stimulation of mesenchymal stem cells and endothelial progenitor cells cocultures in three-dimensional scaffolds under in vitro and in vivo paradigms for vascularized bone tissue engine. Tissue Eng Part A. https://doi.org/10.1089/ten.tea.2012.0187

Liu Y, Chan JKY, Teoh SH (2015) Review of vascularised bone tissue-engineering strategies with a focus on co-culture systems. J Tissue Eng Regen Med 9:85

Lode A, Bernhardt A, Gelinsky M (2008) Cultivation of human bone marrow stromal cells on three-dimensional scaffolds of mineralized collagen: influence of seeding density on colonization, proliferation and osteogenic differentiation. J Tissue Eng Regen Med. https://doi.org/10.1002/term.110

Loi F, Córdova LA, Pajarinen J, Lin T, Yao Z, Goodman SB (2016) Inflammation, fracture and bone repair. Bone 86:119

Ma J, Both SK, Yang F, Cui F-Z, Pan J, Meijer GJ, Jansen JA, van den Beucken JJJP (2014) Concise review: cell-based strategies in bone tissue engineering and regenerative medicine. Stem Cells Transl Med. https://doi.org/10.5966/sctm.2013-0126

Marolt Presen D, Traweger A, Gimona M, Redl H (2019) Mesenchymal stromal cell-based bone regeneration therapies: from cell transplantation and tissue engineering to therapeutic Secretomes and extracellular vesicles. Front Bioeng Biotechnol 7:1–20. https://doi.org/10.3389/fbioe.2019.00352

Marolt D, Augst A, Freed LE, Vepari C, Fajardo R, Patel N, Gray M, Farley M, Kaplan D, Vunjak-Novakovic G (2006) Bone and cartilage tissue constructs grown using human bone marrow stromal cells, silk scaffolds and rotating bioreactors. Biomaterials 27:6138–6149. https://doi.org/10.1016/j.biomaterials.2006.07.015

Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R (1997) Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 138:4456–4462. https://doi.org/10.1210/endo.138.10.5425

Mauney JR, Volloch V, Kaplan DL (2005) Matrix-mediated retention of adipogenic differentiation potential by human adult bone marrow-derived mesenchymal stem cells during ex vivo expansion. Biomaterials 26:6167–6175. https://doi.org/10.1016/j.biomaterials.2005.03.024

Mauney JR, Kirker-Head C, Abrahamson L, Gronowicz G, Volloch V, Kaplan DL (2006) Matrix-mediated retention of in vitro osteogenic differentiation potential and in vivo bone-forming capacity by human adult bone marrow-derived mesenchymal stem cells during ex vivo expansion. J Biomed Mater Res Part A 79:464–475. https://doi.org/10.1002/jbm.a.30876

McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 6:483–495

Melchels FPW, Domingos MAN, Klein TJ, Malda J, Bartolo PJ, Hutmacher DW (2012) Additive manufacturing of tissues and organs. Prog Polym Sci 37:1079

Melke J, Zhao F, Ito K, Hofmann S (2020) Orbital seeding of mesenchymal stromal cells increases osteogenic differentiation and bone-like tissue formation. J Orthop Res. https://doi.org/10.1002/jor.24583

Méndez-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. https://doi.org/10.1038/nature09262

Mendicino M, Bailey AM, Wonnacott K, Puri RK, Bauer SR (2014) MSC-based product characterization for clinical trials: an FDA perspective. Cell Stem Cell 14:141–145

Miao C, Lei M, Hu W, Han S, Wang Q (2017) A brief review: the therapeutic potential of bone marrow mesenchymal stem cells in myocardial infarction. Stem Cell Res Ther 8:242

Mizoguchi T, Pinho S, Ahmed J, Kunisaki Y, Hanoun M, Mendelson A (2014) Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. Dev Cell 29:340

Mizuhashi K, Ono W, Matsushita Y, Sakagami N, Takahashi A, Saunders TL, Nagasawa T, Kronenberg HM, Ono N (2018) Resting zone of the growth plate houses a unique class of skeletal stem cells. Nature. https://doi.org/10.1038/s41586-018-0662-5

Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, Hiratsu E, Suzuki S, Miyauchi-Hara C, Nagoshi N, Sunabori T, Shimmura S, Miyawaki A, Nakagawa T, Suda T, Okano H, Matsuzaki Y (2009) Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J Exp Med 206:2483–2496. https://doi.org/10.1084/jem.20091046

Murgia A, Veronesi E, Candini O, Caselli A, D'Souza N, Rasini V, Giorgini A, Catani F, Iughetti L, Dominici M, Burns JS (2016) Potency biomarker signature genes from multiparametric osteogenesis assays: will cGMP human bone marrow mesenchymal stromal cells make bone? PLoS One. https://doi.org/10.1371/journal.pone.0163629

Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F (2002) Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 46:704–713. https://doi.org/10.1002/art.10118

Muschler GF, Boehm C, Easley K (1997) Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. J Bone Jt Surg A 79:1699–1709. https://doi.org/10.2106/00004623-199711000-00012

Nakamura A, Akahane M, Shigematsu H, Tadokoro M, Morita Y, Ohgushi H, Dohi Y, Imamura T, Tanaka Y (2010) Cell sheet transplantation of cultured mesenchymal stem cells enhances bone formation in a rat nonunion model. Bone. https://doi.org/10.1016/j.bone.2009.08.048

Nguyen LH, Annabi N, Nikkhah M, Bae H, Binan L, Park S, Kang Y, Yang Y, Khademhosseini A (2012) Vascularized bone tissue engineering: approaches for potential improvement. Tissue Eng Part B Rev. https://doi.org/10.1089/ten.teb.2012.0012

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. https://doi.org/10.1016/J.IMMUNI.2010.08.017

Ono N, Ono W, Mizoguchi T, Nagasawa T, Frenette PS, Kronenberg HM (2014a) Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. Dev Cell 29:330–339. https://doi.org/10.1016/j.devcel.2014.03.014

Ono N, Ono W, Nagasawa T, Kronenberg HM (2014b) A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. Nat Cell Biol 16:1157–1167. https://doi.org/10.1038/ncb3067

Oryan A, Alidadi S, Moshiri A, Maffulli N (2014) Bone regenerative medicine: classic options, novel strategies, and future directions. J Orthop Surg Res 9:18

Oryan A, Kamali A, Moshirib A, Eslaminejad MB (2017) Role of mesenchymal stem cells in bone regenerative medicine: what is the evidence? Cells Tissues Organs 204:59–83. https://doi.org/10.1159/000469704

Park D, Spencer JA, Koh BI, Kobayashi T, Fujisaki J, Clemens TL, Lin CP, Kronenberg HM, Scadden DT (2012) Cell stem cell endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. Stem Cell 10:259–272. https://doi.org/10.1016/j.stem.2012.02.003

Petite H, Viateau V, Bensaïd W, Meunier A, De Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G (2000) Tissue-engineered bone regeneration. Nat Biotechnol. https://doi.org/10.1038/79449

Pineault KM, Song JY, Kozloff KM, Lucas D, Wellik DM (2019) Hox11 expressing regional skeletal stem cells are progenitors for osteoblasts, chondrocytes and adipocytes throughout life. Nat Commun 10. https://doi.org/10.1038/s41467-019-11100-4

Pinho S, Lacombe J, Hanoun M, Mizoguchi T, Bruns I, Kunisaki Y, Frenette PS (2013) PDGFRα and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. J Exp Med 210:1351–1367. https://doi.org/10.1084/jem.20122252

Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147. https://doi.org/10.1126/science.284.5411.143

Ponte AL, Marais E, Gallay N, Langonné A, Delorme B, Hérault O, Charbord P, Domenech J (2007) The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem Cells 25:1737–1745. https://doi.org/10.1634/stemcells.2007-0054

Pountos I, Corscadden D, Emery P, Giannoudis PV (2007) Mesenchymal stem cell tissue engineering: techniques for isolation, expansion and application. Injury 38(Suppl 4):S23–S33. https://doi.org/10.1016/s0020-1383(08)70006-8

Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M (2001) Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 344:385. https://doi.org/10.1056/NEJM200102013440516

Raftery RM, Mencía-Castaño I, Sperger S, Chen G, Cavanagh B, Feichtinger GA, Redl H, Hacobian A, O'Brien FJ (2018) Delivery of the improved BMP-2-advanced plasmid DNA within a gene-activated scaffold accelerates mesenchymal stem cell osteogenesis and critical size defect repair. J Control Release. https://doi.org/10.1016/j.jconrel.2018.05.022

Redondo LM, García V, Peral B, Verrier A, Becerra J, Sánchez A, García-Sancho J (2018) Repair of maxillary cystic bone defects with mesenchymal stem cells seeded on a cross-linked serum scaffold. J Cranio-Maxillofac Surg. https://doi.org/10.1016/j.jcms.2017.11.004

Regulski MJ (2017) Mesenchymal stem cells: "Guardians of inflammation". Wounds 29:20–27

Reinisch A, Etchart N, Thomas D, Hofmann NA, Fruehwirth M, Sinha S, Chan CK, Senarath-Yapa K, Seo EY, Wearda T, Hartwig UF, Beham-Schmid C, Trajanoski S, Lin Q, Wagner W, Dullin C, Alves F, Andreeff M, Weissman IL, Longaker MT, Schallmoser K, Majeti R, Strunk D (2015) Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. Blood 125:249–260. https://doi.org/10.1182/blood-2014-04-572255

Robey PG, Kuznetsov SA, Ren J, Klein HG, Sabatino M, Stroncek DF (2015) Generation of clinical grade human bone marrow stromal cells for use in bone regeneration. Bone. https://doi.org/10.1016/j.bone.2014.07.020

Roelofs AJ, Zupan J, Riemen AHK, Kania K, Ansboro S, White N, Clark SM, De Bari C (2017) Joint morphogenetic cells in the adult mammalian synovium. Nat Commun 8:15040. https://doi.org/10.1038/ncomms15040

Rosen CJ, Bouillon R, Compston JE, Rosen V (2013) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 8th Edition. ISBN: 978-1-118-45388-9. https://www.wiley.com/en-us/Primer+on+the+Metabolic+Bone+Diseases+and+Disorders+of+Mineral+Metabolism%2C+8th+Edition-p-9781118453889

Rosen CJ, Bouxsein ML (2006) Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol 2:35–43. https://doi.org/10.1038/ncprheum0070

Roseti L, Parisi V, Petretta M, Cavallo C, Desando G, Bartolotti I, Grigolo B (2017) Scaffolds for bone tissue engineering: state of the art and new perspectives. Mater Sci Eng C 78:1246

Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P (2007) Self-renewing Osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131:324–336. https://doi.org/10.1016/J.CELL.2007.08.025

Sacchetti B, Funari A, Remoli C, Giannicola G, Kogler G, Liedtke S, Cossu G, Serafini M, Sampaolesi M, Tagliafico E, Tenedini E, Saggio I, Robey PG, Riminucci M, Bianco P (2016) No identical "mesenchymal stem cells" at different times and sites: human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels. Stem Cell Rep 6:897–913. https://doi.org/10.1016/j.stemcr.2016.05.011

Sakaguchi Y, Sekiya I, Yagishita K, Ichinose S, Shinomiya K, Muneta T (2009) Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obta. Stem Cells 104:2728–2735. https://doi.org/10.1182/blood-2003-12-4452

Samsonraj RM, Rai B, Sathiyanathan P, Puan KJ, Rötzschke O, Hui JH, Raghunath M, Stanton LW, Nurcombe V, Cool SM (2015) Establishing criteria for human mesenchymal stem cell potency. Stem Cells. https://doi.org/10.1002/stem.1982

Sanchez-Guijo FM, Blanco JF, Cruz G, Muntion S, Gomez M, Carrancio S, Lopez-Villar O, Barbado MV, Sanchez-Abarca LI, Blanco B, Briñon JG, Del Cañizo MC (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 336:501–507. https://doi.org/10.1007/s00441-009-0778-x

Santiesteban DY, Kubelick K, Dhada KS, Dumani D, Suggs L, Emelianov S (2016) Monitoring/imaging and regenerative agents for enhancing tissue engineering characterization and therapies. Ann Biomed Eng 44:750–772. https://doi.org/10.1007/s10439-015-1509-y

Santo VE, Gomes ME, Mano JF, Reis RL (2013) Controlled release strategies for bone, cartilage, and osteochondral engineering-Part ii: challenges on the evolution from single to multiple bioactive factor delivery. Tissue Eng B Rev 19:327

Schallmoser K, Rohde E, Reinisch A, Bartmann C, Thaler D, Drexler C, Obenauf AC, Lanzer G, Linkesch W, Strunk D (2008) Rapid large-scale expansion of functional mesenchymal stem cells from unmanipulated bone marrow without animal serum. Tissue Eng Part C Methods 14:185–196. https://doi.org/10.1089/ten.tec.2008.0060

Schallmoser K, Bartmann C, Rohde E, Bork S, Guelly C, Obenauf AC, Reinisch A, Horn P, Ho AD, Strunk D, Wagner W (2010) Replicative senescence-associated gene expression changes in mesenchymal stromal cells are similar under different culture conditions. Haematologica 95:867–874. https://doi.org/10.3324/haematol.2009.011692

Sensebé L, Gadelorge M, Fleury-Cappellesso S (2013) Production of mesenchymal stromal/stem cells according to good manufacturing practices: a review. Stem Cell Res Ther 4:66

Siddiqui JA, Partridge NC (2016) Physiological bone remodeling: systemic regulation and growth factor involvement. Physiology 31:233

Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG (2003) Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci USA. https://doi.org/10.1073/pnas.2434367100

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. https://doi.org/10.1002/jcp.20238

Solchaga LA, Penick K, Goldberg VM, Caplan AI, Welter JF (2010) Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. Tissue Eng Part A 16:1009–1019. https://doi.org/10.1089/ten.TEA.2009.0100

Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21:70

Stockmann P, Park J, Von Wilmowsky C, Nkenke E, Felszeghy E, Dehner JF, Schmitt C, Tudor C, Schlegel KA (2012) Guided bone regeneration in pig calvarial bone defects using autologous mesenchymal stem/progenitor cells – a comparison of different tissue sources. J Cranio-Maxillofac Surg 40:310–320. https://doi.org/10.1016/j.jcms.2011.05.004

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. https://doi.org/10.1016/j.mad.2007.12.002

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. https://doi.org/10.1016/j.immuni.2006.10.016

Sukul M, Nguyen TBL, Min YK, Lee SY, Lee BT (2015) Effect of local sustainable release of BMP2-VEGF from Nano-cellulose loaded in sponge biphasic calcium phosphate on bone regeneration. Tissue Eng Part A. https://doi.org/10.1089/ten.tea.2014.0497

Szpalski C, Barbaro M, Sagebin F, Warren SM (2012) Bone tissue engineering: current strategies and techniques-Part II: cell types. Tissue Eng Part B Rev 18:258

Tang D, Tare RS, Yang LY, Williams DF, Ou KL, Oreffo ROC (2016) Biofabrication of bone tissue: approaches, challenges and translation for bone regeneration. Biomaterials 83:363

Tarafder S, Lee CH (2017) 3D printing integrated with controlled delivery for musculoskeletal tissue engineering. J 3D Print Med 1:181–189. https://doi.org/10.2217/3dp-2017-0005

Tasoglu S, Demirci U (2013) Bioprinting for stem cell research. Trends Biotechnol 31:10

Tikhonova AN, Dolgalev I, Hu H, Sivaraj KK, Hoxha E, Cuesta-Domínguez Á, Pinho S, Akhmetzyanova I, Gao J, Witkowski M, Guillamot M, Gutkin MC, Zhang Y, Marier C, Diefenbach C, Kousteni S, Heguy A, Zhong H, Fooksman DR, Butler JM, Economides A, Frenette PS, Adams RH, Satija R, Tsirigos A, Aifantis I (2019) The bone marrow microenvironment at single-cell resolution. Nature 569:222–228. https://doi.org/10.1038/s41586-019-1104-8

Timmins NE, Scherberich A, Früh JA, Heberer M, Martin I, Jakob M (2007) Three-dimensional cell culture and tissue engineering in a T-CUP (tissue culture under perfusion). Tissue Eng. https://doi.org/10.1089/ten.2006.0158

Tuli R, Tuli S, Nandi S, Wang ML, Alexander PG, Haleem-Smith H, Hozack WJ, Manner PA, Danielson KG, Tuan RS (2003) Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. Stem Cells 21:681–693. https://doi.org/10.1634/stemcells.21-6-681

Turnbull G, Clarke J, Picard F, Riches P, Jia L, Han F, Li B, Shu W (2018) 3D bioactive composite scaffolds for bone tissue engineering. Bioact Mater 3:278

Uccelli A, Laroni A, Brundin L, Clanet M, Fernandez O, Nabavi SM, Muraro PA, Oliveri RS, Radue EW, Sellner J, Soelberg Sorensen P, Sormani MP, Wuerfel JT, Battaglia MA, Freedman MS, Bonetti B, Rush C, Herrera C, Ramo Tello C, Miller D, Szwajcer D, Strunk D, Wall D, Aguera-Morales E, Rohde E, Dazzi F, Comi G, Martino G, Izquierdo Ayuso G, Rabinovitch H, MacLean H, Marriott J, Racosta J, Arab L, Gimona M, Introna M, Blinkenberg M, Aghdami N, Ali R, Vosoughi R, Nicholas R, Marrie RA, Karimi S (2019) MEsenchymal StEm cells for multiple sclerosis (MESEMS): a randomized, double blind, cross-over phase I/II clinical trial with autologous mesenchymal stem cells for the therapy of multiple sclerosis. Trials 20:263. https://doi.org/10.1186/s13063-019-3346-z

Unger RE, Dohle E, Kirkpatrick CJ (2015) Improving vascularization of engineered bone through the generation of pro-angiogenic effects in co-culture systems. Adv Drug Deliv Rev 94:116

Uth N, Mueller J, Smucker B, Yousefi AM (2017) Validation of scaffold design optimization in bone tissue engineering: finite element modeling versus designed experiments. Biofabrication. https://doi.org/10.1088/1758-5090/9/1/015023

Viswanathan S, Shi Y, Galipeau J, Krampera M, Leblanc K (2019) Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT Ò) mesenchymal stromal cell committee. Cytotherapy 21:1019–1024. https://doi.org/10.1016/j.jcyt.2019.08.002

Volarevic V, Markovic BS, Harrell CR, Fellabaum C, Jovicic N, Djonov V, Arsenijevic N (2019) Use of mesenchymal stem cells in inflammatory bowel disease. Springer, Cham, pp 125–138

Wang Z, Mithieux SM, Weiss AS (2019) Fabrication techniques for vascular and vascularized tissue engineering. Adv Healthc Mater 8:1900742. https://doi.org/10.1002/adhm.201900742

Warnke P, Springer I, Wiltfang PJ, Acil PY, Eufinger PH, Wehmöller M, Russo P, Bolte H, Sherry E, Behrens E, Terheyden PH (2004) Growth and transplantation of a custom vascularised bone graft in a man. Lancet. https://doi.org/10.1016/S0140-6736(04)16935-3

Wei Q, Frenette PS (2018) Niches for hematopoietic stem cells and their progeny. Immunity 48:632–648. https://doi.org/10.1016/J.IMMUNI.2018.03.024

Winkler T, Sass FA, Duda GN, Schmidt-Bleek K (2018) A review of biomaterials in bone defect healing, remaining shortcomings and future opportunities for bone tissue engineering: the unsolved challenge. Bone J Res 7:232

Worthley DL, Churchill M, Compton JT, Tailor Y, Rao M, Si Y, Levin D, Schwartz MG, Uygur A, Hayakawa Y, Gross S, Renz BW, Setlik W, Martinez AN, Chen X, Nizami S, Lee HG, Kang HP, Caldwell JM, Asfaha S, Westphalen CB, Graham T, Jin G, Nagar K, Wang H, Kheirbek MA, Kolhe A, Carpenter J, Glaire M, Nair A, Renders S, Manieri N, Muthupalani S, Fox JG, Reichert M, Giraud AS, Schwabe RF, Pradere JP, Walton K, Prakash A, Gumucio D, Rustgi AK, Stappenbeck TS, Friedman RA, Gershon MD, Sims P, Grikscheit T, Lee FY, Karsenty G, Mukherjee S, Wang TC (2015) Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. Cell 160:269–284. https://doi.org/10.1016/j.cell.2014.11.042

Wu H, Kang N, Wang Q, Dong P, Lv X, Cao Y, Xiao R (2015) The dose–effect relationship between the seeding quantity of human marrow mesenchymal stem cells and in vivo tissue-engineered bone yield. Cell Transplant. https://doi.org/10.3727/096368914X685393

Wuchter P, Vetter M, Saffrich R, Diehlmann A, Bieback K, Ho AD, Horn P (2016) Evaluation of GMP-compliant culture media for in vitro expansion of human bone marrow mesenchymal stromal cells. Exp Hematol 44:508–518. https://doi.org/10.1016/j.exphem.2016.02.004

Zachar L, Bačenková D, Rosocha J (2016) Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment. J Inflamm Res 9:231–240. https://doi.org/10.2147/JIR.S121994

Zadpoor AA (2015) Bone tissue regeneration: the role of scaffold geometry. Biomater Sci 3:231

Zhao Q, Ren H, Han Z (2016) Mesenchymal stem cells: immunomodulatory capability and clinical potential in immune diseases. J Cell Immunother 2:3–20. https://doi.org/10.1016/j.jocit.2014.12.001

Zhao L, Chen S, Yang P, Cao H, Li L (2019) The role of mesenchymal stem cells in hematopoietic stem cell transplantation: prevention and treatment of graft-versus-host disease. Stem Cell Res Ther 10:182. https://doi.org/10.1186/s13287-019-1287-9

Zhou BO, Yue R, Murphy MM, Peyer JG, Morrison SJ (2014) Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. Cell Stem Cell 15:154–168. https://doi.org/10.1016/j.stem.2014.06.008

Zhu H, Guo Z-K, Jiang X-X, Li H, Wang X-Y, Yao H-Y, Zhang Y, Mao N (2010) A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. Nat Protoc 5:550–560. https://doi.org/10.1038/nprot.2009.238

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228

Pancreatic Islet Beta-Cell Replacement Strategies

J. Jason Collier and Susan J. Burke

Contents

Abstract

In this chapter, we outline some of the basic aspects of autoimmunity that leads to the loss of both function and quantity of pancreatic islet β-cells during progression to type 1 diabetes. Then we focus on strategies designed to promote replacement of pancreatic β-cells, either by whole pancreas or islet transplantation from cadaveric donors. In addition, approaches to engineer large populations of β-cells from embryonic or induced pluripotent stem cells as a replacement alterative to cadaveric tissue are considered. Understanding the nuances of the

J. J. Collier (✉)
Basic Sciences/Islet Biology, Pennington Biomedical Research Center, Baton Rouge, LA, USA
e-mail: Jason.collier@pbrc.edu

S. J. Burke
Basic Sciences/Immunogenetics, Pennington Biomedical Research Center, Baton Rouge, LA, USA
e-mail: Susan.collier@pbrc.edu

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_3

endocrine lineage and its developmental features has been supported by mouse genetics, with results showing promise that other endocrine cell types can become "reprogrammed" into insulin-positive cells. Thus, at the present time, several distinct approaches have the potential to drive novel therapeutic interventions designed to replace β-cells lost due to autoimmune mechanisms. In an idyllic scenario, the best approach(es) will offer strong salutary benefit with reduced or no side effects, allowing for long-term (e.g., decades), if not permanent, functioning of the transplanted tissue. The ultimate goal is full correction of hyperglycemia with minimal potential for hypoglycemic events.

1 Introduction

Autoimmunity describes specific conditions where an organism's immune responses become directed against its own cells and tissues. Diseases that arise from such abnormal immune activity are designated as "autoimmune diseases." Well-known examples of such autoimmune diseases include celiac disease, systemic lupus erythematosus (SLE), Sjögren's syndrome, Hashimoto's thyroiditis, Graves' disease, rheumatoid arthritis, and type 1 diabetes mellitus (T1D). Autoimmune diseases are often subclassified into systemic or organ-specific. For example, rheumatoid arthritis is a systemic, while T1D is an organ-specific autoimmune disease. For the purposes of this chapter, we will refer continually to T1D as an example of an autoimmune disease that can be treated by either whole pancreas or pancreatic islet transplantation; variations of these ideas include engineering populations of insulin-producing cells from other (i.e., not donor-derived) sources. Nuances of each of these approaches will also be considered.

T1D results when insulin levels are insufficient to control blood glucose levels due to autoimmune events that reduce the total number, or the normal function, of the insulin-producing pancreatic β-cell (Atkinson et al. 2014). An estimated 1.25 million children and adults have T1D in the United States alone; this number is much higher worldwide. Symptoms that bring individuals into the clinic include frequent urination, excessive thirst, tiredness and lethargy, and in some cases, blurry vision. A series of clinical tests often reveal blood glucose levels greater than 250 mg/dL or higher (normal range 70–100 mg/dL) and a hemoglobin A_{1C} level of $>6.5\%$, which in many cases is often much higher. It is not uncommon for two distinct assays of the aforementioned types to be used for diagnosis of diabetes. While the initial trigger that ultimately results in manifestation of this endocrine disease is unknown, the underlying pathology is immune cell-mediated death and/or dysfunction of the pancreatic islet β-cells (Staeva et al. 2013).

In many different species, the islets of Langerhans produce and release protein hormones that regulate macronutrient metabolism (Arrojo e Drigo et al. 2015). The pancreatic β-cells are the predominant cell type in both rodent and human islets and are responsible for the production and secretion of insulin (Arrojo e Drigo et al. 2015).

Insulin is released from pancreatic β-cells in response to elevations in blood glucose levels, and this response can be modulated by a number of other signals (Rorsman and Ashcroft 2018). The electrical activity of the islet β-cell is an important component of its response to nutrient secretagogues. Glucose triggers action potentials in pancreatic β-cells, with the closing of ATP-gated potassium channels and the opening of voltage-gated calcium channels playing key roles in the trafficking and exocytosis of the packaged insulin granules (Rorsman and Ashcroft 2018). Additional pathways beyond the classic ATP-gated activity exist and are also important for full control of insulin secretion (Henquin 2011).

The hormone insulin promotes glucose uptake into adipose tissue and skeletal muscle and suppresses glucose production from the liver. Thus, insulin is an important hormone signaling the transition from the fasting to the fed state (White and Kahn 1994; Cheatham and Kahn 1995). Loss of insulin, such as occurs in T1D, prevents this physiological transition. Consequently, T1D was observed in the past to be a disease where there was "starvation amidst plenty," signifying that nutrient intake could not be properly managed by the organism in the absence of insulin. Accordingly, the discovery of insulin in 1921 as the critical factor restoring metabolic homeostasis was a lifesaving new therapy and prompted studies into the mechanisms of action of insulin in a variety of tissues over the last century. These initial landmark studies by Frederick Banting and Charles Best, which reported on the "internal secretion of the pancreas" (Banting and Best 1922), became the seminal and Nobel Prize winning breakthrough we now know as the discovery of the hormone insulin (Roth et al. 2012). Insulin injection is still a therapy for T1D in the present day, with a variety of short-, medium-, and long-acting versions now available through molecular biology approaches (Mathieu et al. 2017). Implantable devices and pump-based delivery of the hormone have also been used as additional strategies to promote metabolic homeostasis. However, while lifesaving, many of these approaches do not provide the minute to minute control of blood glucose afforded by endogenous functional pancreatic islets, which has prompted research into optimization of pancreas and islet transplantation as well as strategies to engineering insulin-producing cells from a variety of sources.

While the molecular events leading to organ-specific autoimmunity and ultimately the selective loss of the pancreatic islet β-cell function (leading to insulin insufficiency) are not entirely understood, many key events have emerged via the efforts of numerous investigators over the past century (Fig. 1). It is clear that multiple immune cell types cooperate to selectively target the insulin-producing cells (Lehuen et al. 2010; Wallberg and Cooke 2013). Leukocytes representative of the innate immune (e.g., macrophage, neutrophil, etc.) and the adaptive immune systems (e.g., T-lymphocytes) are involved at different stages of the development and progression of the disease. In addition, these immune cell populations also contribute significantly to the process of tissue graft rejection. The interactions between the distinct immune cell populations and the resident immune cells within the pancreatic islets are still a very active research area. Throughout this chapter, we will attempt to focus, where appropriate, on what is

known about therapeutic interventions designed to treat individuals after they are diagnosed with T1D, i.e., replacement of pancreatic islet β-cells and strategies to generate populations of β-cells from various sources. In particular, we will highlight cellular engineering approaches that aim to improve tissue transplantation outcomes and/or provide novel sources of β-cells to offset the currently limiting factors (e.g., number of donors) to treat T1D by replacing β-cell mass lost due to autoimmune events.

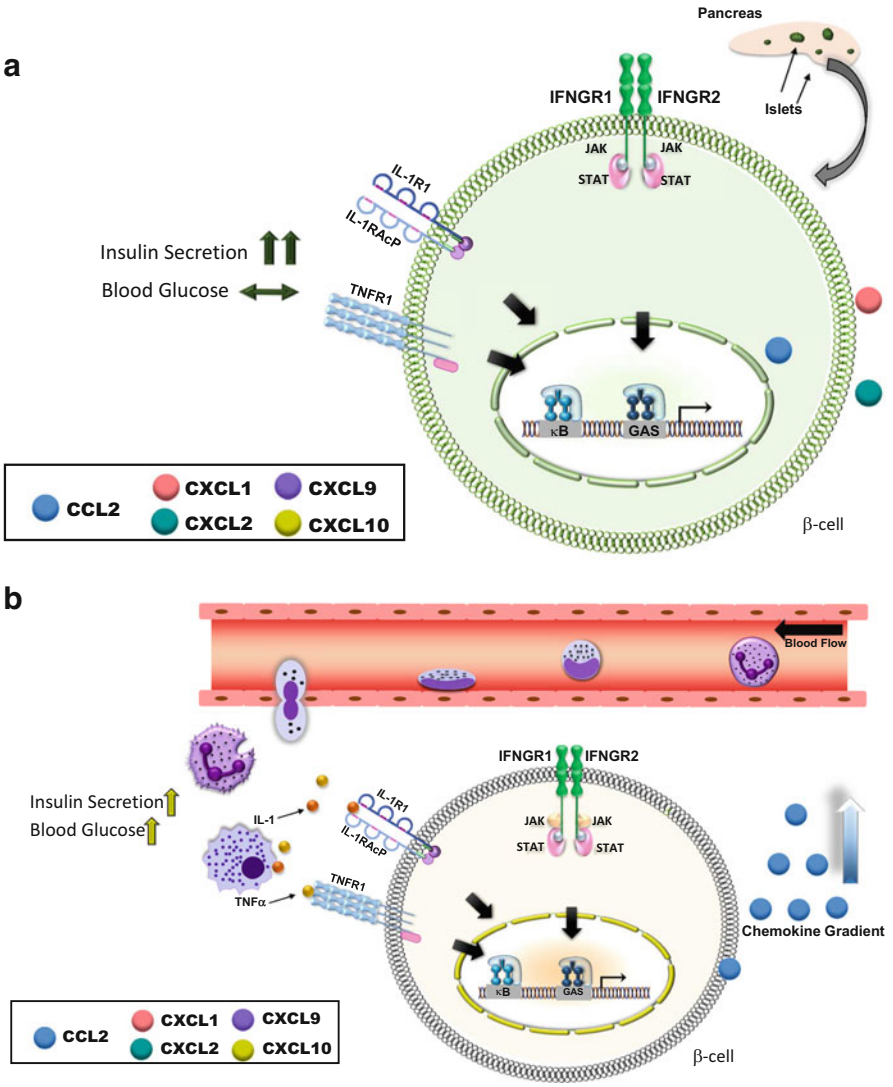


Fig. 1 (continued)

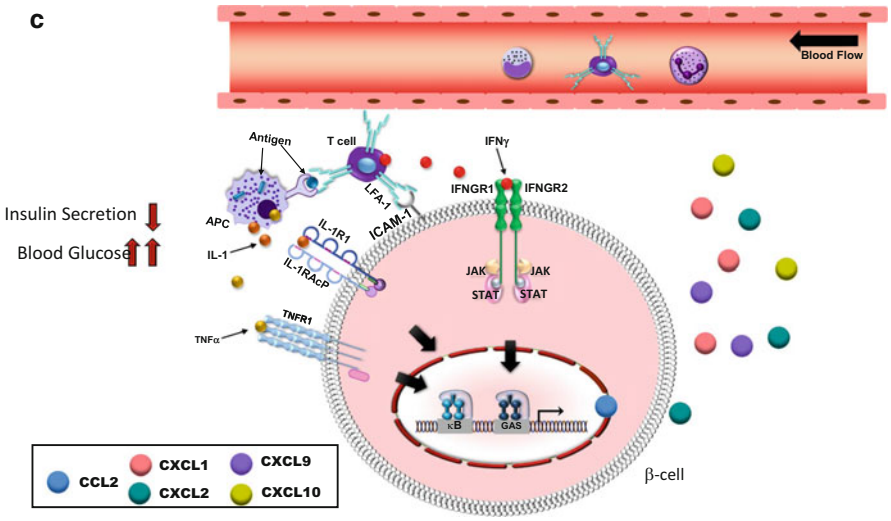


Fig. 1 Simplified mechanisms of pancreatic islet β-cell death and dysfunction due to autoimmunity. (**a**) Top right: Pancreatic tissue showing the islets of Langerhans scattered throughout (not to scale), with the arrow indicating a blown-up view of an individual β-cell (green). The green-colored β-cell indicates that it is still healthy, i.e., secreting insulin in response to the usual array of stimuli present in vivo. Blood glucose would thus be in the normal range (sideways double-headed arrow). The presence of the interleukin-1 receptor (IL-1R1), TNF receptor 1 (TNFR1), and the interferon gamma receptors 1 and 2 (IFNGR1/IFNGR2) shows that cytokine receptors are traditionally present on islet β-cells. A basal level of specific chemokine is typical and may reflect communication of β-cells with resident immune cells. (**b**) Early in the autoimmune process (e.g., multiple autoantibody-positive situations), as well as in tissue graft rejection, cells of the innate immune system begin to infiltrate the pancreatic tissue. These cells can release cytokines, such as IL-1 and TNF-α, which activate inflammatory signaling pathways within the β-cell. Insulin secretion may be decreased (denoted by yellow β-cell) relative to organisms with no autoimmune events, with abnormal glucose levels detected via fasting samples or by glucose tolerance testing. (**c**) Once T-lymphocytes start to intrude into the pancreatic tissue, they interact with both antigen-presenting cells (APC) and ICAM-1 on β-cells via lymphocyte function-associated antigen 1 (LFA-1) on the T-cell surface. Multiple cytokines are now activating both NF-κB and STAT1 pathways within β-cells, leading to marked reductions in insulin secretion and greater production of chemokines from β-cells (red color). Once insulin secretion is insufficient to regulate blood glucose levels properly, clinical diagnosis of T1D is imminent

2 The Pancreas Contains the Islets of Langerhans, Which Are Small Micro-organs that Regulate Macronutrient Homeostasis

If I have ever made any valuable discoveries, it has owed more to patient attention, than to any other talent. – Sir Isaac Newton[1]

[1]History, N. M. M. o. S. (accessed 5/29/2018). "http://www.nmspacemuseum.org/halloffame/detail.php?id=139."

The above paraphrased quote, which has been attributed to Sir Isaac Newton regarding his discoveries in science and mathematics, is also pertinent in the realms of islet biology and islet transplantation. These fields have benefitted from the careful attention to detail shown by numerous investigators in their pursuit of advancing from a strategy of subcutaneous implants of sheep's pancreatic tissue into an adolescent with ketoacidosis (Williams 1894) to achieving full insulin independence in patients several years after transplantation of isolated islets (Fiorina et al. 2008). The pancreatic islets are only 1–2% of the total mass of the pancreatic tissue and are thus incredibly minute in terms of total body mass. Consequently, it is quite amazing how important their role is in the context of glucose homeostasis and, when not functional, the eventual onset of metabolic disease. In addition, the islets have a remarkable ability to respond and adapt to a variety of situations that reduce insulin sensitivity in the peripheral tissues (Burke et al. 2016). Thus, the restoration of islet function, or discovery of replacement options for islet cells, to prevent or treat diabetes is a major focus of many research programs worldwide. Herein, we will give a brief overview of pancreatic transplantation, its distinction from islet transplantation, followed by strategies to enhance success of islets isolated, purified, and implanted.

3 Pancreas Versus Islet Transplantation

A number of different methods for transplanting pancreatic tissue or tissue fragments have been attempted over the last century. These studies involved many different species, including mouse, rat, pig, and dog. The most advantageous approaches were then translated to human subjects. The technical details are of interesting historical significance because each experience provided the scientific community with new insights and inspiration to explore a variety of technical nuances. The best strategy to preserve tissue isolated from the donor prior to transplant into the recipient has long been sought out. For those interested in the historical achievements, and the associated narratives, the reader is referred to both older (Lillehei et al. 1970a, b) and more recent literature (Gruessner and Gruessner 2013a).

4 Pancreas Transplantation

We are using the term "pancreas transplantation" to include any surgical procedure that results in a donor pancreas being transferred into a recipient. This includes simultaneous pancreas-kidney, pancreas after kidney, and "pancreas alone" transplantation procedures. The simultaneous pancreas and kidney transplant is indicated for subjects with T1D that have progressed to chronic kidney disease as a result of hyperglycemic damage to the glomeruli. In this case, the glomeruli increase in size with physiological changes that include protein loss via the urine. The estimated glomerular filtration rate (eGFR) often decreases progressively from a normal range to a point where the patient displays features of end-stage kidney disease (ESKD). It

is estimated that close to 80% of pancreas transplants fall into this simultaneous pancreas-kidney category (Israni et al. 2014). While the pancreas transplantation procedures can be conducted for reasons other than treating T1D (e.g., to replace exocrine pancreas function, etc.), this is typically less common. The vast majority of pancreas transplants are specifically to treat patients with T1D.

The "pancreas after kidney" transplantation procedure is performed as the name suggests: after a living-donor kidney transplantation has already occurred. The procedure has been reported to be declining over time and only accounts for 10–12% of current pancreas transplants (Israni et al. 2014). Pancreas transplants alone are also less common than the simultaneous pancreas-kidney procedure, accounting for the remaining 8–10% of procedures (Israni et al. 2014). The pancreas alone transplant is often indicated when T1D patients are having extreme difficulty controlling their blood glucose levels with other means and are at severe risk for recurring hypoglycemic episodes (Robertson et al. 2006). However, these patients typically have normal renal function.

Regardless of the procedure being conducted (of the three aforementioned types), the organs are typically procured from brain-dead donors. A variety of factors specific to the donor impact the success of the tissue to be transplanted; these include age, BMI, medical history, etc. Organs from younger donors and with lower BMI typically offer higher success rates (Axelrod et al. 2010). Not surprisingly, recipient BMI >25 is often associated with poorer graft success (Humar et al. 2000). The reasons for poorer outcomes in overweight and obese patients are numerous but involve increased strain on islets because of the likelihood, or increased risk, of peripheral insulin resistance in subjects with BMI >25. In addition, excess adipose tissue leads to increased production of cytokines and other molecules that could influence glucose homeostasis (Kershaw and Flier 2004; Grant and Dixit 2015). On the other hand, the positives of pancreas transplantation are that T1D patients with recurring and substantial hypoglycemic events display markedly improved quality of life. In addition, the pancreas transplantation procedure has one of the highest success rates, in terms of patient survival, compared with other organ transplant procedures (Gruessner and Gruessner 2013a, b). However, this strategy differs from the transplantation of islets, which are isolated from whole pancreas and then transplanted in the absence of exocrine tissue (Shapiro et al. 2017).

5 Transplantation of Isolated Pancreatic Islets

> For people unable to safely control type 1 diabetes despite optimal medical management, islet transplantation offers hope for improving not only physical health but also overall quality of life. – Dr. Anthony Fauci, director of the NIAID[2]

[2]NIH.gov (accessed 5/30/2018). "https://www.nih.gov/news-events/news-releases/islet-transplantation-improves-quality-life-people-hard-control-type-1-diabetes."

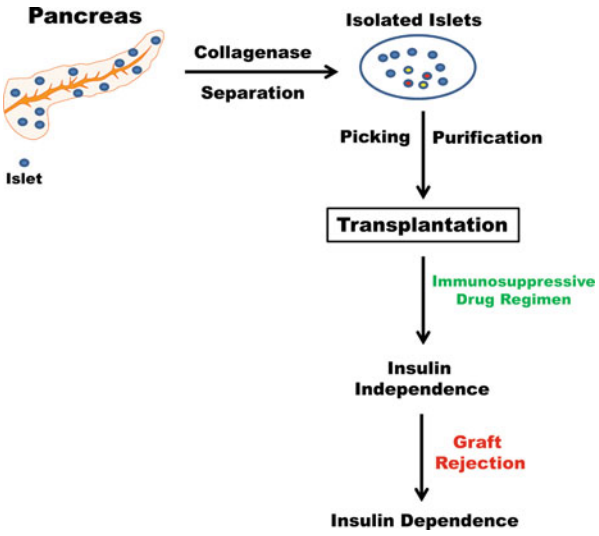


Fig. 2 General summary of islet isolation and transplantation. Whole pancreatic tissue is subjected to collagenase digestion (in some cases this enzymatic digestion is accompanied by mechanical digestion) followed by recovery in culture for a designated period of time. The isolated islets are then handpicked from the remaining pancreatic components (e.g., acinar tissue, ductal cells, fibroblasts, etc.). These handpicked islets are used for transplantation in both rodents (for research purposes) and human subjects (for therapeutic outcomes). Immunosuppressive drug regimens are required for most situations, with transplanted tissue ideally producing insulin independence. Unfortunately, this independence from injected insulin is not permanent, and upon graft rejection, there is a dependence on exogenously administered insulin

A procedure distinct from the pancreas transplantation options delineated above is direct delivery of islets that have been isolated from pancreatic tissue. Figure 2 provides a diagrammatic overview of this procedure within the workflow of transplantation. The rise in popularity of this technique is likely due to its relatively straightforward nature as a surgical procedure coupled with its immense potential benefit to the patient. While the nuances of islet isolation have evolved, the basis of this process has stayed remarkably similar to the original report from Paul Lacy's group (Lacy and Kostianovsky 1967). Briefly, this process involves the infusion of highly purified enzymes (i.e., collagenase) into the pancreatic bile duct which runs through the majority (e.g., head, body, and tail) of the pancreas. Once infused throughout the pancreatic tissue, enzymatic digestion begins to promote separation of acinar tissue from the endocrine tissue (i.e., islets of Langerhans).

In humans, the pancreas is subsequently cut into small chunks and transferred into a specialized chamber, originally developed by Dr. Camillo Ricordi, where digestion is facilitated by mechanical means (Ricordi et al. 1988a, b). Isolated islets are then handpicked from the remaining exocrine tissue and other remaining cellular debris. For transplantation, these newly isolated and purified islets are transferred into the hepatic portal vein via catheterization (Scharp et al. 1990). Immunosuppressive

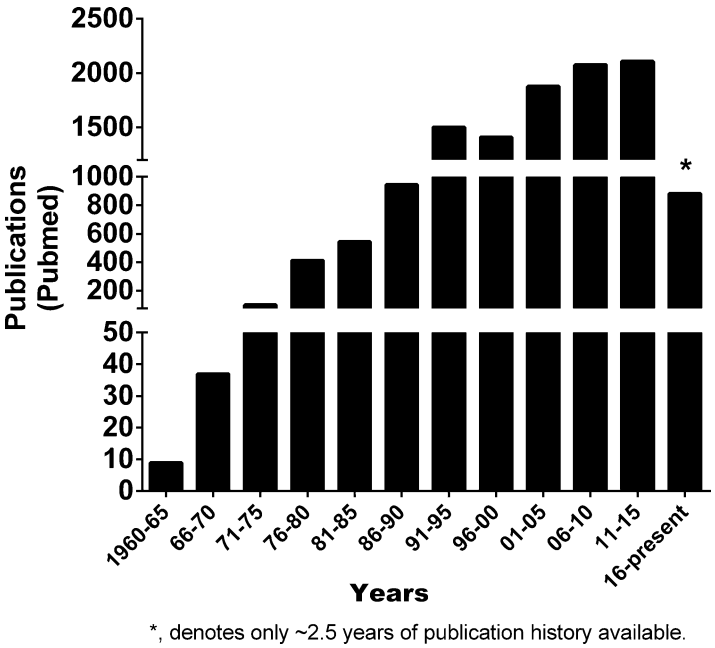


Fig. 3 Increase in publications relevant to islet transplantation. Searches using the phrase "islet transplantation" were conducted and broken down into 5-year periods to illustrate increased interest and focus in this area over the past few decades. Note that the asterisk above the last column indicates that only 2.5 years of the most recent available data were plotted, whereas all the other columns represent 5 years of data

agents, many of which had been used previously to prevent rejection after other organ transplants, have subsequently also been used in islet transplantation to reduce the likelihood of graft rejection. Some individuals do achieve insulin independence with this strategy, but most revert back to dependence on exogenously administered insulin at some point during their lifetime. The rise in publications relevant to "islet transplantation" over the last 60+ years (Fig. 3) is a testament to the dedication of the research community in trying to find a suitable and long-term solution to the growing public health problem of diabetes, with islet transplantation constituting a major focus.

6 Preclinical Models of Islet Transplantation

Any discussion of seminal contributions to islet transplantation procedures should include the key discoveries of Dr. Paul Lacy. As noted above, his lab developed a procedure for isolating islets from rats (Lacy and Kostianovsky 1967), which is remarkable not only for its breakthrough in islet biology but also because it is still in use by many laboratories in the present day. Subsequently, Lacy and colleagues further determined that these isolated islets, which retained their ability to secrete insulin in

response to glucose in culture, could be transplanted under the kidney capsule in rats. The recipient rats were made diabetic by the chemical compound streptozotocin (STZ), followed by correction of hyperglycemia by islet transplantation (Ballinger and Lacy 1972). STZ is a chemical that structurally resembles a glucose analogue, allowing it to be transported into β-cells via the GLUT2 transporter (Szkudelski 2001). Once taken up by β-cells, STZ produces alkylating DNA damage, resulting in death of the insulin-producing cells that ultimately leads to hyperglycemia, polyuria, etc. Thus, the STZ model of hyperglycemia is often used as a model of T1D because it can be easily and reproducibly generated in the rodent, offering an opportunity to study mechanisms of islet transplantation and other strategies to lower blood glucose levels. In the pioneering studies overseen by Dr. Lacy, the donor islets were initially from normal (i.e., nondiabetic) rats that were genetically matched; thus, this is considered a syngeneic transplant. This is distinct from allogeneic transplantation approaches, wherein a non-genetically matched donor is used. In the case of allogeneic transplants, immunosuppressive regimens are often necessary to prevent or reduce the likelihood of the donor tissue being rejected by the recipient.

In further progress on islet transplantations using the streptozotocin model, Lacy and coworkers also transplanted islets into the hepatic portal vein (Kemp et al. 1973). The significance of the liver transplant site is momentous for two key reasons. The first is that it provided a site where insulin secretion would directly support glucose intake into hepatocytes, which is important because the liver is one of the most responsive tissues to the hormone insulin. In addition, glucose metabolism in the liver directly influences whole-body glucose homeostasis (Collier and Scott 2004). The second key point is that the intraportal site became the location of choice when islet transplantation was moved from preclinical studies to human clinical trials (Scharp et al. 1990). Importantly, this is still the site of islet transplantation in human recipients in current medical practice (Shapiro et al. 2017). Thus, Lacy and colleagues were at the forefront of islet transplantation studies and have shaped the way this technique is conducted in modern medicine.

To address limitations with the success of human islet transplantation, many investigators returned to the preclinical models in attempts to understand why grafts failed and to devise strategies that may be useful in clinical medicine. Because of the large variety of genetic manipulations and lower costs of housing, many laboratories began to use the mouse for studies on mechanisms of graft rejection, number of islets required to achieve euglycemia, and alternate sites of transplantation. In the following sections, we will detail a number of achievements that have been made through the use of new technologies coupled with investigations into mouse models of islet transplantation.

7 Detection of β-Cell Loss in T1D and After Islet Transplantation

In any study of transplanted tissue, determining whether the tissue of interest survived the transplantation procedure is of particular interest and importance to the success of the intervention. Thus, strategies designed to detect islet β-cell death

have been sought. One of the newer methods developed to detect β-cell death using a noninvasive approach involves droplet digital polymerase chain reaction (ddPCR). In this technique, serum isolated from animals or human patients is used to isolate cell-free DNA (cfDNA). This cfDNA is then subjected to bisulfite conversion prior to detection of DNA quantities that serve as a proxy for beta-cell death. Exposure of DNA to bisulfite changes the nitrogenous base cytosine residues to uracil, while methylated cytosines (e.g., 5-methylcytosine) are protected. Consequently, DNA exposed to the bisulfite retains the methylated cytosines, which can be useful in determining from which tissue the DNA originated. This is useful because most, if not all, tissues methylate the promoter regions of the DNA within the insulin gene to keep them from being expressed in non-β-cells. Conversely, because β-cells produce insulin at high levels, the DNA of the insulin promoter region from within β-cells is not methylated. Thus, sequencing or ddPCR can be carried out to determine the quantity of cfDNA that likely originated from dying β-cells. This technique has started to gain traction in both animal studies (Juliana et al. 2017) and human clinical trials (Fisher et al. 2015; Herold et al. 2015). It is noteworthy that additional assays using detection of methylated and unmethylated regions of DNA controlling expression of amylase or glucokinase are also being suggested as appropriate targets to detect β-cell death (Olsen et al. 2016; Sklenarova et al. 2017). Finally, while in its infancy for studies relevant to pancreatic islets, the ddPCR technique is now being used to detect loss of β-cells that occurs during islet procurement, as well as death that occurs after transplantation (Bellin et al. 2017).

8 Cellular and Tissue Engineering Approaches to Generate New β-Cells

While optimizing the immunosuppressive drug regimen is still an ongoing goal to support continued efforts to improve pancreas and islet transplantation outcomes, another major limitation is the shortage of donor pancreata. The number of donors is vastly smaller than the number of individuals with T1D that are candidates for transplantation. In addition, with the number of islets needed for any one recipient and the knowledge that true insulin independence requires repeated islet transplants, either a change in recipient priority or strategies to engineer larger quantities of transplantable β-cells will be required to meet the demand. We will address the former point briefly prior to addressing the latter point in more detail below.

There is currently a supply and demand issue where the number of patients waiting for organ donation far exceeds the available organs needed for transplantation (Girlanda 2016). This issue is not specific to pancreas or islet transplantation, but also extends to other organs to be donated. In the case of islet transplantation, the criteria and priority that are likely to be most useful in determining which candidates to select for the procedure include the following considerations. First, frequent and severe hypoglycemic events are common indications where islet transplantation is highly effective (Ludwig et al. 2010). Second, patients with a good history of adherence and compliance with medical management are often seen as excellent candidates. Third, when there is an unusual or sooner than normal appearance of

complications associated with T1D, islet transplantation is often an excellent therapeutic strategy.

A final noteworthy point is that any new β-cells transplanted into a patient with T1D will be subject to the same autoimmune attack as the original cells. Therefore, strategies to protect these transplanted cells will need to be refined. Whether macroencapsulation, microencapsulation, or drugs that counteract the immune responses are used, the health and function of the β-cells is paramount to the success of any new therapeutic modality.

8.1 Engineered Cell Lines

The establishment of a variety of rodent cell lines, derived from both mouse and rat, used to study β-cell function has been achieved by numerous investigators [reviewed in (Hohmeier and Newgard 2004)]. The challenge has been getting primary β-cells to proliferate, with human β-cells especially refractory to many different approaches, especially when the expectation is that these endocrine cells maintain their highly differentiated state. Methods that are successful in promoting β-cell replication typically have the negative side effect of inducing a less differentiated state, often with loss of expression of key transcription factors controlling insulin production and secretion. A number of different strategies have been employed in various attempts to provide a greater number of islets for transplantation purposes as well as to circumvent the need for, or to improve upon, existing immunosuppression regimens. Many of these were either not successful, yielded insights into the relationship between proliferation and insulin secretion, or have produced some degree of advancement in the field. We will briefly highlight some of the approaches present in the literature, emphasizing notable achievements relevant to human β-cells whenever possible.

Initial excitement ensued over the reported development of a bona fide human β-cell line that would serve both research and possible transplantation purposes. In 2005, Narushima described a strategy to expand an existing population of human β-cells (Narushima et al. 2005). In this approach, the investigators delivered SV40 large T-antigen along with human telomerase reverse transcriptase. Both genes were bordered by loxP sites to effect downstream removal by cre recombinase. To start, the investigators first tested 271 cell lines that arose from this approach for their ability to grow tumors in immunodeficient mice; 253 of these lines did not display tumorigenic potential. They next screened the designated non-tumorigenic engineered cells for expression of insulin as well as key transcription factors necessary to maintain adult β-cell function. Of this starting value of 253, only one line, labeled NAKT-15, displayed criteria consistent with human β-cells. For example, these cells appeared to secrete insulin in the physiological concentration range and demonstrated responsiveness to additional stimuli, including sulfonylureas, glucagon-like peptide 1, and fatty acids. Moreover, when these cells were transplanted into diabetic mice, control of blood glucose was maintained out to 7 months or longer. Blood glucose was reported to be stable with no documented spikes in either low (hypoglycemia) or high (hyperglycemia) glycemic ranges. While this Cre/

loxP-based reversible immortalized pancreatic beta-cell clone certainly has potential, we have not seen any reported use of the NAKT-15 cell line beyond the initial publication (Narushima et al. 2005).

Additional approaches that have emerged to generate human β-cell lines include electrofusion of human islet β-cells with an immortalized cell line called PANC-1. PANC-1 cells, an epithelioid cell line, are ductal-based pancreatic carcinomas that were originally established in culture for research purposes (Lieber et al. 1975). Using the electrofusion approach, McCluskey and colleagues reported on three novel human insulin-secreting cell lines, which they term 1.1B4, 1.4E7, and 1.1E7 (McCluskey et al. 2011). When the 1.1B4 cell line was transplanted into streptozotocin-treated immunodeficient mice (to make them hyperglycemic), blood glucose levels were lowered. However, insulin levels in these transplanted cells were less than what is traditionally observed within primary human β-cells. Thus, further strategies have been examined by other investigators.

Alternative strategies to producing a human β-cell line with properties similar to primary human β-cells have reported by Scharfmann and coworkers (Ravassard et al. 2011). The approach taken was conceptually similar to that by Narushima, in that the SV40 large T-antigen (SV40LT) was used to drive immortalization, but fetal tissue was the starting material instead of adult human tissue. The rationale is that fetal tissue might be more "malleable" in terms of transformation when compared with highly differentiated adult tissue. Importantly, the investigators validated the approach first using rat tissue (Ravassard et al. 2009). Following the generation of an insulinoma, via the SV40LT transduction with lentiviral vectors, they introduced human telomerase reverse transcriptase. The cells were then placed into immunodeficient mice where they matured into adult tissue, followed by excision and further growth in culture. The resulting cells were designated EndoC-βH1 (Ravassard et al. 2011).

These new human β-cells were found to express the critical transcription factors that drive insulin production and also to support stimulus secretion coupling of insulin secretion to metabolic effectors. While these cells have been characterized by a number of investigators [see (Scharfmann et al. 2016; Tsonkova et al. 2018) and references therein], the results have not all been positive. For example, at least one report indicates that EndoC-βH1 cells do not retain characteristics of human islets in studies of cytokine signaling (Oleson et al. 2015). This is important because pro-inflammatory cytokines are considered to be a critical component of the autoimmune response that results in T1D. Nontheless, at the present time, the EndoC-βH1 cells appear to be gaining traction as a model for human β-cells, although further studies will be needed to determine whether these cells are as good as it gets or whether additional improvements emerge in the field of human β-cell lines.

8.2 β-Cells Derived from ES Cells

A stem cell is an early progenitor type of cell dedicated to the purpose of giving rise to other cell types through precise differentiation pathways. The embryonic stem (ES) cell has the specific ability and potential to mature and progress into discrete

cell types in an organism, which is absolutely required during normal development and eventual tissue growth that occurs during the progression to the adult organism. In the generation of a new organism, it is the stem cells within a developing embryo, initially termed a blastocyst, that ultimately give rise to distinct tissues. In the fully developed adult organism, stem cells can also serve an additional role to help renew, or replenish, dead and dysfunctional cells within individual tissues. As such, ES cells provide an internal mechanism for replacing cells during the lifespan. Stem cells therefore hold the potential to become virtually any cell type depending on external cues they receive. For reference genes that may indicate or distinguish a stem cell from a terminally differentiated or non-pluripotent cell, gene signatures have been reported (Ivanova et al. 2002; Ramalho-Santos et al. 2002). Given these unique formative abilities, populations of stem cells may thus provide unique opportunities to generate lost tissue that, *once regenerated*, would offer a treatment for diabetes, heart disease, as well as many other chronic conditions attributed to loss of specialized cell function (e.g., β-cells, cardiomyocytes, hepatocytes, etc.). This field of research is often placed under the heading of regenerative medicine and offers seemingly limitless potential once the biology of each individual developmental process can be unlocked.

While multiple avenues for engineering β-cell populations from ES cell starting material have been documented [e.g., see (Johnson 2016)], we will outline some of the early seminal work in this area. One of the first reports describing the generation of insulin-producing cells from a mouse ES cell population involved using a genetic "cell-trapping" system, which selected for insulin-positive cells by providing a resistance gene (e.g., hygromycin) driven within the desired population by the insulin promoter (Soria et al. 2000). These cells were allowed to form clusters or aggregates by culturing in non-treated dishes (normal cells are grown on tissue culture-treated dishes which allow them to adhere). These aggregates were then transplanted into the spleens of male Swiss mice made diabetic by a single high-dose injection of streptozotocin. The implantation of these ES cell-derived insulin-producing cells normalized blood glucose of the diabetic mice within 1 week. While mice made diabetic will exhibit reduced total body mass over a period of weeks, the mice receiving the ES cell-derived transplanted cells were able to increase their body weight, consistent with steady delivery of insulin in vivo. Thus, clear evidence of engineering a desired cell population directly from ES cells with a positive therapeutic impact on the disease state in an experimental animal was achieved.

Lumelsky and coworkers soon followed with their own distinct strategy in 2001 (Lumelsky et al. 2001). In this approach, the investigators used a five-stage scheme that was adapted from earlier reports on the ES-based generation of neural cells (Lee et al. 2000). In the first stage, the ES cell population is expanded but not allowed to differentiate into any particular cell type. In the second stage, embryoid body formation, which is essentially a cluster of ES cells and other pluripotent stem cells, was used to select for nestin-positive cells. Nestin had been previously found to be associated with a subpopulation of hormone-negative pancreatic cells, which could be isolated for further study (Zulewski et al. 2001). In the third stage, the

nestin-positive population was enriched by culture in serum-free medium, which allowed the population of non-nestin-positive cell types to die off.

In the fourth stage, the nestin-positive population was enhanced in number by the inclusion of the specific signaling molecule basic fibroblast growth factor (bFGF; aka FGF2) to augment cell proliferation. In the fifth stage, FGF2 was removed with the concomitant simultaneous addition of a supplement termed B-27, a reagent typically used to support neuronal cell growth, and also the micronutrient nicotinamide. This procedure was reported to yield numerous aggregates of cells, of which greater than 30% express insulin (Lumelsky et al. 2001). While the investigators did not find markers of pancreatic exocrine tissue using this procedure, the insulin-positive cells generated using this multistep approach contained 50 times less insulin per cell relative to β-cells within the normal mouse islet. In addition, these cells did not support long-term regulation of blood glucose levels once transplanted into mice. However, it is worth noting that a subcutaneous site of transplantation was used, which may not have been ideal for correction of hyperglycemia in their model.

8.3 β-Cells Derived from iPSCs

While the most established stem cell population is the embryonic stem cell (described above), there are other cell types that have analogous, if not equivalent, stem cell properties. The induced pluripotent stem cell (iPSC) is derived from an adult cell type rather than from the preimplantation phase embryo. The iPSC population has the advantage that they can be obtained from individual patients so that individualized or personalized medicine becomes possible; thus, the risk of tissue rejection from cells engineered from the iPSC starting point is vastly reduced and ideally eliminated. The generation of a pluripotent stem cell population from adult somatic cells was first described by Takahashi and Yamanaka (2006). This milestone in stem cell biology was achieved by introducing transcription factors, typically associated with embryonic stem cells, into fibroblasts and selecting for cells that obtained ES cell-like properties. Starting with 24 target genes, associated with various properties of ES cells (e.g., pluripotency, self-renewal, etc.), Takahashi and Yamanaka narrowed the list down to four genes that could be introduced to revert an adult cell to a pluripotent state: c-Myc, Klf4, Oct3/Oct4, and Sox2. Importantly, the authors went on to show that these iPSCs had the ability to form embryoid bodies in culture and to form teratomas in mice, all properties associated with ES cells. However, while transcriptional analysis showed that the iPSCs were more similar to ES cells than to the original fibroblast population, they did retain a distinct gene expression pattern. Thus, iPSCs are a separate population of pluripotent cell when compared with true ES cells, which may be one reason they are less controversial for scientific study.

Using mouse embryonic fibroblasts as a starting source material, a report described the combination of lineage-specific signals, in combination with the same strategy of generating iPSCs as that used by Takahashi and Yamanaka, to

generate definitive endoderm-like cells (termed DELCs) as a novel strategy to deal with the problem of engineering large quantities of insulin-producing cells (Li et al. 2014). Using the DELCs, the investigators next set out to isolate precise culture conditions that would produce pancreatic progenitor-like cells (PPLCs) with high expression of two key transcription factors controlling β-cell differentiation: Nkx6.1 and Pdx1. Nkx6.1 is one of the transcription factors which specifies β-cell development and also helps to maintain adult β-cell phenotype (Miralles et al. 1999; Oliver-Krasinski and Stoffers 2008; Schaffer et al. 2013). In addition to specifying pancreatic tissue development, Pdx1 also helps to support maturation into endocrine tissue and plays an important role in establishing mature β-cell function (Sander and German 1997; Oliver-Krasinski and Stoffers 2008).

It was determined that, out of 400 chemicals screened, retinoic acid, a TGF-β receptor inhibitor, a modified vitamin C derivative (2-phospho-L-ascorbic acid), and a hedgehog pathway inhibitor routed the progenitor cells toward greater expression of Nkx6.1 and Pdx1 (Li et al. 2014). This approach was used to then show that downstream inhibition of the p38 MAP kinase, in combination with additional delivery of 2-phospho-L-ascorbic acid, promoted expression of insulin. The presence of other endocrine cell population markers, including glucagon, somatostatin, and amylase (acinar-like cells), was also noted. The authors point out that, in each endocrine-like cell, only the hormone specific to that cell was being produced, which is an important defining characteristic of mature endocrine cell populations (Li et al. 2014). When further examining the insulin-positive cell population, it was determined that these cells did release both insulin and C-peptide in response to glucose stimulation. In addition, the sulfonylurea drug tolbutamide promoted insulin release as well as the often used IBMX, which raises intracellular cAMP levels. However, these β-like cells did not achieve the level of insulin secretion produced by isolated mouse islets.

The gold standard for determining β-cell function from such a differentiation procedure has been the ability of such cells to return a preclinical diabetic mouse to the normoglycemia state. As discussed above, the streptozotocin-induced mouse or rat is often used for this purpose. In line with this in vivo approach, Li et al. indeed showed that the endocrine cells produced from their PPLC strategy did indeed show the ability to regulate blood glucose levels in a diabetic model after transplantation. Importantly, they compared these new endocrine cells with blood glucose regulation provided by the transplantation of mouse islets (Li et al. 2014). Another intriguing component of β-cells generated by this iPSC approach is that these cells, once extracted from the in vivo model, demonstrated a mostly mono-hormonal state with bihormonal cells only infrequently detected. Thus, the approach clearly demonstrates that generation of functional insulin-producing cells is feasbile using mouse embryonic fibroblasts as a starting point. Consequently, this strategy offers hope that perhaps a similar method could be used to generate new β-cells from human subjects using their own fibroblasts as the source.

8.4 β-Cells Derived from Transdifferentiation

Because multiple cell types are present within the pancreas as well as within the pancreatic islets, it is reasonable to speculate that some of these non-insulin-producing cell populations may be able to give rise to insulin-producing cells under specific conditions. Thus, this is defined as transdifferentiation, a process that defines the conversion of one differentiated cell type into a distinct differentiated cell type clearly distinguishable from the original starting cell. Thorel and colleagues tested this hypothesis directly using several mouse genetic models, coupled with a selective reduction of the β-cell population (Thorel et al. 2010), to determine the potential of converting one endocrine cell type (e.g., α-cell) into another (e.g., β-cell). This innovative strategy consisted of generating mice with diphtheria toxin receptor driven by the rat insulin promoter which allows selective destruction of the β-cell population upon administration of diphtheria toxin. The authors tested whether, in this severe model of β-cell destruction (>99% loss of insulin-positive cells), new β-cells arose from replication of residual β-cells. Whereas β-cells normally arise through self-duplication in other mouse models (Dor et al. 2004; Sharma et al. 2015), in this quite drastic model of β-cell ablation by diphtheria toxin, the authors found that β-cells were not replenishing via replication of existing β-cells (Thorel et al. 2010). Indeed, lineage tracing showed that while the population of labeled β-cells decreased dramatically (from 80% labeled to 8% labeled), the total β-cell mass increased by threefold. Thus, they concluded that the formation of new β-cells to replace the population destroyed by diphtheria toxin was derived from non-β-cell sources.

The authors next used lineage tracing approaches, again via mouse genetics, to determine the source of the new β-cells. In one model, glucagon promoter sequences drive expression of the rtTA to ensure alpha-cell specificity, coupled with TetO-Cre/R26-YFP, to allow for inducible labeling. Taking this approach, Thorel and colleagues determined that 90% of α-cells were labeled once doxycycline (dox) was administered to the mice. After 2 weeks of dox exposure to induce the label, the diphtheria toxin-specific reduction of β-cells was repeated. Rapidly after decreasing the β-cell population using the diphtheria approach, these authors found that 90% of the YFP-labeled cells, which were originally α-cells, were now positive for both glucagon and insulin. This finding indicated that α-cells are capable of being reprogrammed to express insulin under situations of extreme β-cell loss. They were able to further confirm these results with additional genetic approaches, consistent with the overall conclusion that α-cells can give rise to β-cells under specific conditions. However, there was no evidence to suggest that the β-cells, once newly derived from α-cells, start to proliferate to expand the β-cell population. Consequently, whether these new β-cells are terminally differentiated or still in a quasi-differentiated state is not clear.

It is noteworthy that additional work, following up on the idea that α-cells can acquire β-cell like phenotypes, showed that loss of aristaless-related homeobox (Arx) and DNA methyltransferase 1 (Dnmt1) allowed the original glucagon-positive

cells to acquire MafA expression, production of insulin, and electrical properties associated with β-cells (Chakravarthy et al. 2017). Thus, Arx and Dnmt1 clearly help to maintain the differentiated α-cell phenotype, and when their expression is reduced or lost completely, a transition to a more insulin-like state becomes possible. Interestingly, the authors demonstrated that subpopulations of glucagon-expressing cells from T1D patients also lost expression of Arx and Dnmt1; as a result, these human islet cells acquired expression of insulin and other genes associated with β-cells (Chakravarthy et al. 2017). In a separate study, but also of note, is the ability of somatostatin-producing cells within the pancreatic islets to become insulin-positive cells (Chera et al. 2014), thus indicating that multiple endocrine cell types may have the ability to replace β-cells under select conditions of β-cell loss. Whether these approaches operate in the context of autoimmunity is not known, although the data are promising because they indicate potential to interconvert endocrine cell types under select conditions. Importantly, until a bona fide cure for T1D is established, the collective information from these reprogramming approaches are useful in comparison with, or to inform, methods being established for ES and iPS cells.

9 Conclusions, Final Comments, and Future Directions

In its present form, islet transplantation from cadaveric donors to need-based recipients is not a cure for T1D. For example, when patients are treated at centers *known for their islet transplantation expertise*, graft function usually only persists no longer than 5 years after surgery. Thus, less experienced centers may not have this level of success. Graft function is usually defined by C-peptide production in excess of 0.5 ng/mL (Ryan et al. 2005). The downside is that 10% or less of these patients maintain insulin independence out to the 5-year mark (Ryan et al. 2005). Problems that are currently viewed as impediments to the success of islet transplantation include revascularization of the transplanted tissue and complications associated with the present immunosuppressive drug regimens. By its nature, the enzymatic digestion of pancreatic tissue and ensuing procedures used to separate islets from exocrine tissue disrupt the normal vascular connections. After transplantation, the islets need to become vascularized successfully to initiate and retain proper function. Experimental testing has revealed that the revascularization processes occur within the first few days after transplantation and, if successful, reestablish islet function by 10–14 days (Menger et al. 1989; Zhang et al. 2004). These concerns, in addition to the problem of limited tissue availability in the organ donor pool, have led to efforts to generate β-cells from alternative sources, such as stem cells or transdifferentiation of a non-β-cell into a β-cell. In addition, regardless of the source of replacement β-cells (e.g., pancreas transplant, islet transplant, stem cells, etc.), the autoimmunity problem, the issue of graft rejection, vascularization, and improvements to the immunosuppressive drug therapy are still outstanding scientific and medical problems that need a solution. Possible solutions may come from new small molecule therapeutics, such as novel glucocorticoid receptor agonists or from antibody-based approaches that prevent immune cell activity toward the transplanted cells or tissues.

Combination therapies may eventually offer the most promising and advantageous outcomes. Ideally, the best approach will offer strong salutary benefit with reduced or no side effects, allowing for long-term (e.g., decades), if not permanent, functioning of the transplanted tissue.

References

Arrojo e Drigo R, Ali Y, Diez J, Srinivasan DK, Berggren PO, Boehm BO (2015) New insights into the architecture of the islet of Langerhans: a focused cross-species assessment. Diabetologia 58 (10):2218–2228

Atkinson MA, Eisenbarth GS, Michels AW (2014) Type 1 diabetes. Lancet 383(9911):69–82

Axelrod DA, Sung RS, Meyer KH, Wolfe RA, Kaufman DB (2010) Systematic evaluation of pancreas allograft quality, outcomes and geographic variation in utilization. Am J Transplant 10 (4):837–845

Ballinger WF, Lacy PE (1972) Transplantation of intact pancreatic islets in rats. Surgery 72 (2):175–186

Banting FG, Best CH (1922) The internal secretion of the pancreas. J Lab Clin Med 7(5):465–480

Bellin MD, Clark P, Usmani-Brown S, Dunn TB, Beilman GJ, Chinnakotla S, Pruett TL, Ptacek P, Hering BJ, Wang Z, Gilmore T, Wilhelm JJ, Hodges JS, Moran A, Herold KC (2017) Unmethylated insulin DNA is elevated after total pancreatectomy with islet autotransplantation: assessment of a novel beta cell marker. Am J Transplant 17(4):1112–1118

Burke SJ, Karlstad MD, Collier JJ (2016) Pancreatic islet responses to metabolic trauma. Shock 46 (3):230–238

Chakravarthy H, Gu X, Enge M, Dai X, Wang Y, Damond N, Downie C, Liu K, Wang J, Xing Y, Chera S, Thorel F, Quake S, Oberholzer J, MacDonald PE, Herrera PL, Kim SK (2017) Converting adult pancreatic islet alpha cells into beta cells by targeting both Dnmt1 and Arx. Cell Metab 25(3):622–634

Cheatham B, Kahn CR (1995) Insulin action and the insulin signaling network. Endocr Rev 16 (2):117–142

Chera S, Baronnier D, Ghila L, Cigliola V, Jensen JN, Gu G, Furuyama K, Thorel F, Gribble FM, Reimann F, Herrera PL (2014) Diabetes recovery by age-dependent conversion of pancreatic delta-cells into insulin producers. Nature 514(7523):503–507

Collier JJ, Scott DK (2004) Sweet changes: glucose homeostasis can be altered by manipulating genes controlling hepatic glucose metabolism. Mol Endocrinol 18(5):1051–1063

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(6987):41–46

Fiorina P, Shapiro AM, Ricordi C, Secchi A (2008) The clinical impact of islet transplantation. Am J Transplant 8(10):1990–1997

Fisher MM, Watkins RA, Blum J, Evans-Molina C, Chalasani N, DiMeglio LA, Mather KJ, Tersey SA, Mirmira RG (2015) Elevations in circulating methylated and unmethylated preproinsulin DNA in new-onset type 1 diabetes. Diabetes 64(11):3867–3872

Girlanda R (2016) Deceased organ donation for transplantation: challenges and opportunities. World J Transplant 6(3):451–459

Grant RW, Dixit VD (2015) Adipose tissue as an immunological organ. Obesity (Silver Spring) 23 (3):512–518

Gruessner RW, Gruessner AC (2013a) The current state of pancreas transplantation. Nat Rev Endocrinol 9(9):555–562

Gruessner RW, Gruessner AC (2013b) Pancreas transplant alone: a procedure coming of age. Diabetes Care 36(8):2440–2447

Henquin JC (2011) The dual control of insulin secretion by glucose involves triggering and amplifying pathways in beta-cells. Diabetes Res Clin Pract 93(Suppl 1):S27–S31

Herold KC, Usmani-Brown S, Ghazi T, Lebastchi J, Beam CA, Bellin MD, Ledizet M, Sosenko JM, Krischer JP, Palmer JP, Type 1 Diabetes TrialNet Study Group (2015) β cell death and dysfunction during type 1 diabetes development in at-risk individuals. J Clin Invest 125 (3):1163–1173

History NMMoS. http://www.nmspacemuseum.org/halloffame/detail.php?id=139. Accessed 29 May 2018

Hohmeier HE, Newgard CB (2004) Cell lines derived from pancreatic islets. Mol Cell Endocrinol 228(1–2):121–128

Humar A, Kandaswamy R, Granger D, Gruessner RW, Gruessner AC, Sutherland DE (2000) Decreased surgical risks of pancreas transplantation in the modern era. Ann Surg 231 (2):269–275

Israni AK, Skeans MA, Gustafson SK, Schnitzler MA, Wainright JL, Carrico RJ, Tyler KH, Kades LA, Kandaswamy R, Snyder JJ, Kasiske BL (2014) OPTN/SRTR 2012 annual data report: pancreas. Am J Transplant 14(Suppl 1):45–68

Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR (2002) A stem cell molecular signature. Science 298(5593):601–604

Johnson JD (2016) The quest to make fully functional human pancreatic beta cells from embryonic stem cells: climbing a mountain in the clouds. Diabetologia 59(10):2047–2057

Juliana CA, Yang J, Rozo AV, Good A, Groff DN, Wang SZ, Green MR, Stoffers DA (2017) ATF5 regulates beta-cell survival during stress. Proc Natl Acad Sci USA 114(6):1341–1346

Kemp CB, Knight MJ, Scharp DW, Lacy PE, Ballinger WF (1973) Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. Nature 244(5416):447

Kershaw EE, Flier JS (2004) Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89 (6):2548–2556

Lacy PE, Kostianovsky M (1967) Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 16(1):35–39

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(6):675–679

Lehuen A, Diana J, Zaccone P, Cooke A (2010) Immune cell crosstalk in type 1 diabetes. Nat Rev Immunol 10(7):501–513

Li K, Zhu S, Russ HA, Xu S, Xu T, Zhang Y, Ma T, Hebrok M, Ding S (2014) Small molecules facilitate the reprogramming of mouse fibroblasts into pancreatic lineages. Cell Stem Cell 14 (2):228–236

Lieber M, Mazzetta J, Nelson-Rees W, Kaplan M, Todaro G (1975) Establishment of a continuous tumor-cell line (panc-1) from a human carcinoma of the exocrine pancreas. Int J Cancer 15 (5):741–747

Lillehei RC, Simmons RL, Najarian JS, Goetz FC (1970a) Pancreatico-duodenal and renal allotransplantation in juvenile onset, insulin dependent, diabetes mellitus with terminal nephropathy. Langenbecks Arch Chir 326(2):88–105

Lillehei RC, Simmons RL, Najarian JS, Weil R, Uchida H, Ruiz JO, Kjellstrand CM, Goetz FC (1970b) Pancreatico-duodenal allotransplantation: experimental and clinical experience. Ann Surg 172(3):405–436

Ludwig B, Ludwig S, Steffen A, Saeger HD, Bornstein SR (2010) Islet versus pancreas transplantation in type 1 diabetes: competitive or complementary? Curr Diab Rep 10(6):506–511

Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R (2001) Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292 (5520):1389–1394

Mathieu C, Gillard P, Benhalima K (2017) Insulin analogues in type 1 diabetes mellitus: getting better all the time. Nat Rev Endocrinol 13(7):385–399

McCluskey JT, Hamid M, Guo-Parke H, McClenaghan NH, Gomis R, Flatt PR (2011) Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion. J Biol Chem 286(25):21982–21,992

Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K (1989) Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. Diabetes 38(Suppl 1):199–201

Miralles F, Serup P, Cluzeaud F, Vandewalle A, Czernichow P, Scharfmann R (1999) Characterization of beta cells developed in vitro from rat embryonic pancreatic epithelium. Dev Dyn 214 (2):116–126

Narushima M, Kobayashi N, Okitsu T, Tanaka Y, Li SA, Chen Y, Miki A, Tanaka K, Nakaji S, Takei K, Gutierrez AS, Rivas-Carrillo JD, Navarro-Alvarez N, Jun HS, Westerman KA, Noguchi H, Lakey JR, Leboulch P, Tanaka N, Yoon JW (2005) A human beta-cell line for transplantation therapy to control type 1 diabetes. Nat Biotechnol 23(10):1274–1282

NIH.gov. https://www.nih.gov/news-events/news-releases/islet-transplantation-improves-quality-life-people-hard-control-type-1-diabetes. Accessed 30 May 2018

Oleson BJ, McGraw JA, Broniowska KA, Annamalai M, Chen J, Bushkofsky JR, Davis DB, Corbett JA, Mathews CE (2015) Distinct differences in the responses of the human pancreatic beta-cell line EndoC-betaH1 and human islets to proinflammatory cytokines. Am J Physiol Regul Integr Comp Physiol 309(5):R525–R534

Oliver-Krasinski JM, Stoffers DA (2008) On the origin of the beta cell. Genes Dev 22 (15):1998–2021

Olsen JA, Kenna LA, Spelios MG, Hessner MJ, Akirav EM (2016) Circulating differentially methylated amylin DNA as a biomarker of beta-cell loss in type 1 diabetes. PLoS One 11(4): e0152662

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

Ravassard P, Emilie B-N, Hazhouz Y, Pechberty S, Mallet J, Czernichow P, Scharfmann R (2009) A new strategy to generate functional insulin-producing cell lines by somatic gene transfer into pancreatic progenitors. PLoS One 4(3):e4731

Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P, Scharfmann R (2011) A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. J Clin Invest 121(9):3589–3597

Ricordi C, Finke EH, Dye ES, Socci C, Lacy PE (1988a) Automated isolation of mouse pancreatic islets. Transplantation 46(3):455–457

Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW (1988b) Automated method for isolation of human pancreatic islets. Diabetes 37(4):413–420

Robertson RP, Davis C, Larsen J, Stratta R, Sutherland DE, American Diabetes Association (2006) Pancreas and islet transplantation in type 1 diabetes. Diabetes Care 29(4):935

Rorsman P, Ashcroft FM (2018) Pancreatic beta-cell electrical activity and insulin secretion: of mice and men. Physiol Rev 98(1):117–214

Roth J, Qureshi S, Whitford I, Vranic M, Kahn CR, Fantus IG, Dirks JH (2012) Insulin's discovery: new insights on its ninetieth birthday. Diabetes Metab Res Rev 28(4):293–304

Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM (2005) Five-year follow-up after clinical islet transplantation. Diabetes 54(7):2060–2069

Sander M, German MS (1997) The beta cell transcription factors and development of the pancreas. J Mol Med (Berl) 75(5):327–340

Schaffer AE, Taylor BL, Benthuysen JR, Liu J, Thorel F, Yuan W, Jiao Y, Kaestner KH, Herrera PL, Magnuson MA, May CL, Sander M (2013) Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. PLoS Genet 9(1):e1003274

Scharfmann R, Didiesheim M, Richards P, Chandra V, Oshima M, Albagli O (2016) Mass production of functional human pancreatic beta-cells: why and how? Diabetes Obes Metab 18 (Suppl 1):128–136

Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L, Marchetti P, Gingerich RL, Jaffe AS, Cryer PE et al (1990) Insulin independence after islet transplantation into type I diabetic patient. Diabetes 39(4):515–518

Shapiro AM, Pokrywczynska M, Ricordi C (2017) Clinical pancreatic islet transplantation. Nat Rev Endocrinol 13(5):268–277

Sharma RB, O'Donnell AC, Stamateris RE, Ha B, McCloskey KM, Reynolds PR, Arvan P, Alonso LC (2015) Insulin demand regulates beta cell number via the unfolded protein response. J Clin Invest 125(10):3831–3846

Sklenarova J, Petruzelkova L, Kolouskova S, Lebl J, Sumnik Z, Cinek O (2017) Glucokinase gene may be a more suitable target than the insulin gene for detection of beta cell death. Endocrinology 158(7):2058–2065

Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F (2000) Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes 49(2):157–162

Staeva TP, Chatenoud L, Insel R, Atkinson MA (2013) Recent lessons learned from prevention and recent-onset type 1 diabetes immunotherapy trials. Diabetes 62(1):9–17

Szkudelski T (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50(6):537–546

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

Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL (2010) Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 464(7292):1149–1154

Tsonkova VG, Sand FW, Wolf XA, Grunnet LG, Kirstine Ringgaard A, Ingvorsen C, Winkel L, Kalisz M, Dalgaard K, Bruun C, Fels JJ, Helgstrand C, Hastrup S, Oberg FK, Vernet E, Sandrini MPB, Shaw AC, Jessen C, Gronborg M, Hald J, Willenbrock H, Madsen D, Wernersson R, Hansson L, Jensen JN, Plesner A, Alanentalo T, Petersen MBK, Grapin-Botton A, Honore C, Ahnfelt-Ronne J, Hecksher-Sorensen J, Ravassard P, Madsen OD, Rescan C, Frogne T (2018) The EndoC-betaH1 cell line is a valid model of human beta cells and applicable for screenings to identify novel drug target candidates. Mol Metab 8:144–157

Wallberg M, Cooke A (2013) Immune mechanisms in type 1 diabetes. Trends Immunol 34 (12):583–591

White MF, Kahn CR (1994) The insulin signaling system. J Biol Chem 269(1):1–4

Williams P (1894) Notes on diabetes treated with extract and by grafts of sheep's pancreas. BMJ 2:1303–1304

Zhang N, Richter A, Suriawinata J, Harbaran S, Altomonte J, Cong L, Zhang H, Song K, Meseck M, Bromberg J, Dong H (2004) Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 53(4):963–970

Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50 (3):521–533

The Role of Schwann Cells in Peripheral Nerve Function, Injury, and Repair

Christina M. A. P. Schuh, Ana Maria Sandoval-Castellanos, Cristian De Gregorio, Pamina Contreras-Kallens, and John W. Haycock

Contents

Abstract

The specialized role of the Schwann cell is reviewed in the context of peripheral nerve, spanning neural development, anatomy, signaling, and function. A particular focus of this chapter is the increasingly important role identified in many studies of Schwann cells in nerve injury and repair. We summarize a range of key studies describing these specialized roles, which include the alignment of

C. M. A. P. Schuh (✉) · C. De Gregorio · P. Contreras-Kallens
Centro de Medicina Regenerativa, Facultad de Medicina, Clínica Alemana – Universidad del Desarrollo, Santiago, Chile
e-mail: Schuhcschuh@udd.cl; cmap.schuh@gmail.com; crdegregorio@udd.cl; pamina.ck@gmail.com

A. M. Sandoval-Castellanos · J. W. Haycock
Department of Materials Science and Engineering, The University of Sheffield, Sheffield, UK
e-mail: amsandovalcastellanos1@sheffield.ac.uk; j.w.haycock@sheffield.ac.uk

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_5

Schwann cells along myelinated axons; the protection of signaling pathways between the ganglion bodies, linking the spine and target muscles; and the secretion of growth factors. Myelin structures are reviewed and their organization as nodes for physical protection and as structures that increase action potential velocities of motor and sensory systems. We focus on nerve injury, and mechanisms of nerve wound healing and repair, considering the role of the Schwann cell as it dedifferentiates, proliferates, and redifferentiates. The physical role of the glia in guiding axon regeneration and the role of neurotrophins that communicate via paracrine receptor-mediated signals are considered. Bioengineering strategies are also considered, with innovations in biomaterial scaffolds as medical devices for peripheral nerve repair, with a focus on new technologies and models for evaluation, plus new methods for Schwann and stem cell therapies.

1 Introduction

In the mid-nineteenth century, Theodor Schwann discovered that the external membrane sheathing peripheral nerve axons was composed of separate cells (Koehler et al. 2000). These cells (subsequently named after him – Schwann cells (SC)) and their remarkable ability to wrap around axons were confirmed when electron microscope resolution allowed the visualization of the myelin sheath (Bunge 1986). In the first half of the 1900s, investigators observed that when a nerve from the peripheral nervous system (PNS) was resected, Schwann cells proliferated and migrated to the site of injury, suggesting a potential role in peripheral nerve regeneration (Masson 1932). In 1965, Cravioto proposed a possible explanation for this observation: during embryonic development of the PNS, Schwann cells proliferate and accumulate near the newly formed axons to subsequently wrap around them (Cravioto 1965; Wiley-Livingston and Ellisman 1980). Schwann cell precursors (SCP) and immature Schwann cells were described in newborn animals, where it was found that these cells were essential for PNS development by providing trophic support to sensory and motor developing neurons that have not yet reached a target (Riethmacher et al. 1997; Jessen and Mirsky 2005). Discovering that the SCP phenotype differed from that of mature Schwann cells shed light on one of the main mechanisms by which Schwann cells assisted in regenerating resected axons. Schwann cells transdifferentiate into a phenotype similar to that of SCP, after losing axonal contact (Jessen and Mirsky 2005; Sherman et al. 1993; Dupin et al. 2003). The specialized repair SC, nowadays called Büngner cells, are known to secrete neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT3), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF). They also secrete cytokines and express cell adhesion molecules, thus creating a microenvironment that favors axonal regrowth and nerve regeneration (Jessen and Mirsky 2005, 2016; Doetsch 2003; Bhatheja and Field 2006; Bandtlow et al. 1987; Meyer et al. 1992).

2 Schwann Cells in Peripheral Nerve Development, Anatomy, and Function

Schwann cells are the supportive glial cells within the peripheral nervous system and form the protective neurilemma around the axon nerve fibers. They produce a basal lamina which determines the polarity of Schwann cells, as well as myelinating axons (Simons and Trotter 2007). Schwann cells adhere to the axonal membrane by molecules of the nectin-like family (Necl or SynCAM) (Simons and Trotter 2007). Furthermore, they are responsible for producing and assembling the myelin sheath between nerve fibers and the neurilemma. The presence of the myelin sheath enhances the conduction speed of action potentials, through the formation of specialized nodes of Ranvier (Saladin 2014; Arancibia-Carcamo and Attwell 2014). These discontinuous structures are physically organized such that voltage-gated sodium and potassium ion channels are enriched only at these positions and function according to saltatory (or jumping) nerve conduction (Poliak and Peles 2003). This response is unique to myelinated axons as myelin serves as an electrical insulator. This decreases the capacitance of the axon membrane and consequently membrane electrical resistance across the internode intervals. The nodes of Ranvier are organized as gaps in the myelin sheath, exposing small but regular positions of the underlying neuronal cell membrane, which are clearly visible using low magnification electron microscopy (Poliak and Peles 2003). Myelin is a differentiation product also found in the central nervous system (CNS). However, CNS myelin is produced by oligodendrocytes and differs in biochemical composition compared to that in the PNS. In both systems, it has insulating functions, the dry mass of which is approximately 70% lipid and 30% protein. Myelin is therefore a hydrated lipoprotein and contains around 40% water (Kahan and Moscarello 1985; Chen et al. 2017). The protein component is myelin basic protein, responsible for the formation of compact myelin. PNS myelin also contains protein zero, responsible for forming and maintaining the concentric ring structures of the glial cell membrane that constitute the myelin sheath (Martini and Schachner 1997) (Fig. 1). The myelin nodes are therefore formed as a series of specialized structures and serve to propagate sodium ion diffusion through the axoplasm to the next adjacent myelinated internodes (and in turn the next node of Ranvier). This results in axon potential signaling that is unidirectional. The internodal spaces therefore determine saltatory conduction, where the signal jumps rapidly from node to node. This results in action potential velocities that are ten times faster compared to unmyelinated axons (Stampfli 1954).

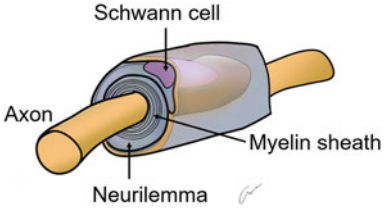


Fig. 1 Schwann cell forming a concentric myelin sheath and neurilemma structure around an axon in the PNS

3 Markers for Schwann Cells

3.1 Early Developmental Markers

One of the most important functions of Schwann cells is phenotypic plasticity, which are tightly regulated by transcriptional networks controlling proliferation, differentiation, dedifferentiation, and myelination (Jessen and Mirsky 2002). The stages of Schwann differentiation have been widely characterized. The embryonic phase of Schwann lineage can be divided in three transient cell stages (Jessen and Mirsky 2002): (i) neural crest cells (NCCs), which start migrating at embryonic day 12 (E12, mouse); (ii) Schwann cell precursors (SCPs), which arise at E12/13 in mouse development and populate the dorsal and ventral roots, the ganglions, and growing nerves (Balakrishnan et al. 2016); and (iii) immature Schwann cells (iSCs), which subsequently differentiate into myelinating or non-myelinating cells, depending on the diameter of the axon to which they are associated (Blanchard et al. 1996; Taveggia and Bolino 2018). The molecular pattern associated with each stage overlaps in part between populations; however, a consensus exists that some molecules correlate with specific developmental stages (reviewed in Jessen and Mirsky 2005). For example, SCPs express the brain fatty acid binding protein (BFABP), desert hedgehog (DHH), and cadherin 19, which are not present in NCCs. In contrast, NCCs do not interact with axons, while SCPs and iSCs associate intimately with axons. Furthermore, SCPs display a dependence on axonal neuregulin-1 (NRG1) for survival (Meier et al. 1999). The main phenotypic markers expressed by Schwann cells during development in the mouse have been described in detail by Balakrishnan et al. (2016).

Schwann cell cultures in vitro have a proliferative phenotype, characterized by a variety of markers, including p75NTR, S100β, SOX10, SOX9, AP2A1, EGR1, PAX3, SOX2, CX32, DHH, NECL4, NFATC4, POU3F1, and YY1 (Stratton et al. 2017). Although studied much less, the effect of aging is thought to modify the pattern and response to injury. It has been shown that aged Schwann cells display a reduced response to injury, such as a decreased c-jun activation and p75 NTR expression compared to young cells in mice (Painter et al. 2014). Similarly, non-myelinating Schwann cells from dental pulp show declines in GAP43 and P75 NTR levels in older mice. Myelinating Schwann cells also have a lower level of myelin basic protein compared to younger mice (Couve et al. 2018).

3.2 Myelination Markers

Transcriptional network studies revealed that Schwann cells are genetically programmed to myelinate. Classical regulators studied in detail include Krox20, Sox10, Oct-6, and NF-κB (Jessen and Mirsky 2008; Stolt and Wegner 2016). The myelination process is dependent on larger-diameter axons, where axonal neuregulin-1 is thought to be a key factor for inducing myelination (Taveggia et al. 2005). Krox20 has been proposed as a major myelin regulator, promoting

cell cycle exit and activating the myelination transcriptional factors Oct-6 and Brn-2 (Jessen and Mirsky 2008; Stolt and Wegner 2016). Sox10 is another transcription factor involved in myelination, though associated with early phases of proliferation and differentiation (Britsch et al. 2001). In contrast, Oct-6 is only required for the initial steps of myelin signaling, with Krox20 and Sox10 responsible for terminal myelin formation and maintenance (Ryu et al. 2007; Bremer et al. 2011).

Schwann cells are able to switch off myelination and reenter to cell cycle when physical axon-Schwann contact is lost following injury. This includes a down-regulation of major myelin molecules, including protein zero, myelin basic protein, and myelin-associated glycoprotein (MAG) (Jessen and Mirsky 2008). Among the most important genes involved in demyelination are Sox2 (Le et al. 2005; Roberts et al. 2017), c-jun (Parkinson et al. 2008), and Pax3 (Doddrell et al. 2012), whose expression is required to maintain Schwann cells in an immature state and exert a reciprocal inhibition to Krox20 (Parkinson et al. 2008). Other complementary factors associated with demyelination include Notch, Krox-24, and Egr-3, which participate in inhibiting pro-myelinating Krox20 signaling and promoting the expression of p75NTR, respectively (Woodhoo et al. 2009; Gao et al. 2007). Thus, an understanding of mechanisms associated with myelination and demyelination has contributed significantly to understanding regulators in differentiation and dedifferentiation during development and in response to injury.

4 Schwann Cells and Their Role Following Nerve Injury

One of the most important roles of Schwann cells is supporting nerve regeneration after injury (Saladin 2014). When an injury affects 90% of the axons within a nerve, Wallerian degeneration occurs, followed by axonal regeneration and end-organ reinnervation (Menorca et al. 2013) (Fig. 2). During Wallerian degeneration, a microenvironment is formed to encourage axonal growth. Initially, Schwann cells recruit macrophages to the site of the injury and phagocytose cell debris. Macrophages secrete pro-inflammatory cytokines, e.g., IL-1 and TNF-α, that encourage dedifferentiation and proliferation of Schwann cells. After the nerve microenvironment is cleared of debris, Schwann cells undergo a redifferentiation program, promoting the expression of neurotrophic factors (e.g., NGF and BDNF) and accelerating differentiation into so-called Büngner Schwann cells in an autocrine manner. The differentiated Büngner Schwann cells also start to physically migrate into the remaining endoneurial tubes distal to the injury and begin to align themselves to form bands of Büngner. Axons emerging from the growth cone proximal of the injury elongate along these bands of Büngner until reinnervation of the target organ. Axonal outgrowth is in part controlled by the Büngner Schwann cells through upregulation of neurotrophic factors. These small molecular weight soluble proteins diffuse and through paracrine signaling stimulate axonal growth, cell survival, and further differentiation of Schwann cells.

Later in the repair process, Schwann cells produce extracellular matrix (ECM) proteins, in particular laminin and fibronectin. Axon-membrane-integrin interactions

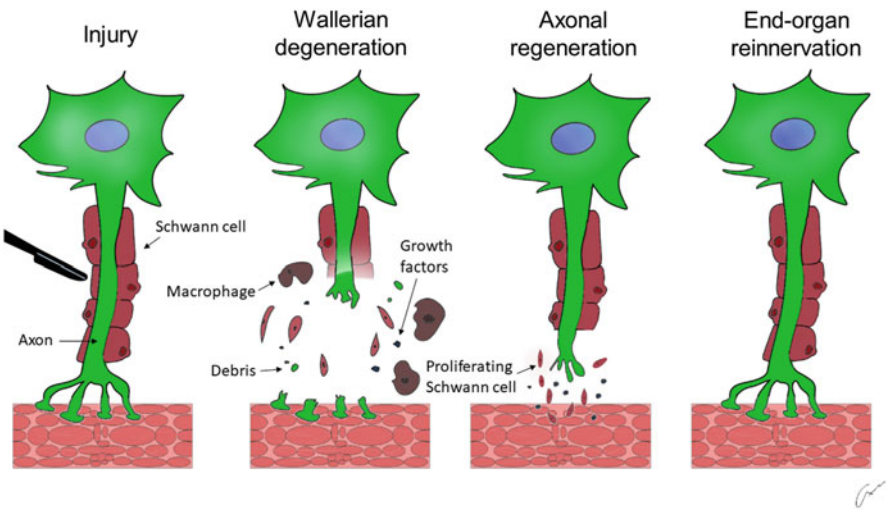


Fig. 2 Nerve regeneration process after injury in the PNS. After the nerve is severed, Schwann cells phagocytose cell debris and recruit macrophages to the site of the injury (Wallerian degeneration). Schwann cells align to form bands of Büngner, creating a path for the axons to regenerate. Finally, the axons reinnervate the target organ

are utilized by the growth cones to physically adhere to the basal lamina of the endoneurial tubes (Menorca et al. 2013; Chiono and Tonda-Turo 2015; Stoll et al. 2002; Frostick et al. 1998; Murray-Dunning et al. 2011). The axonal outgrowth process produces adenosine triphosphate (ATP) and acetylcholine, which encourages reversion of the Schwann cell phenotype, from a proliferating to a myelinating form. A maturation process is then initiated, which includes axon elongation; remyelination, which is also supported by proteins of the ECM (Chernousov et al. 2008); and ultimately functional reinnervation. Functional recovery is not always achieved because these processes are limited to small injury gap distances (typically <5 mm) and the physiological state of the endoneurial tube. If the endoneurial tube is not intact, the formation of bands of Büngner is impeded, and the formation of scar tissue will likely arise (Menorca et al. 2013; Jiang et al. 2010).

4.1 Methods for the Isolation of Schwann Cells

Due to the importance of Schwann cells in peripheral nerve regeneration, the demand for robust and reliable Schwann cell cultures for in vitro testing as well as for cell therapy applications has significantly increasing over the last 10–20 years.

Approaches utilizing cell therapy have found unequivocally that the local delivery of Schwann cells to a wound site (typically within a nerve guide device) improves nerve regeneration (Guenard et al. 1992; Hadlock et al. 2000). This is reported as being primarily due to the secretion of neurotrophic factors (e.g., NGF

and BDNF) and the presence of physical and topographic cues responsible for promoting axonal regeneration (Ide 1996). However, the benefits reported for stimulating nerve repair are impeded in practical terms by the clinical need to use autologous Schwann cells and in particular limited technically due to the very slow proliferation rate of primary Schwann cells in culture. The clinical requirement to sacrifice a healthy donor nerve adds to this practical limitation (Kaewkhaw et al. 2012). The latter requirement is typically complicated as a whole nerve (e.g., the sensory sural nerve) is necessary to source sufficient Schwann cells and additionally creates a surgical donor morbidity site. In an attempt to address this issue, methods have been developed to establish whether more efficient and selective approaches are possible for Schwann cell isolation and culture in vitro.

4.2 Methods for Obtaining and Culturing Schwann Cells

Most methods to culture Schwann cells include a pre-degeneration stage, where Schwann cells are stimulated to dedifferentiate and proliferate in situ from a fresh adult nerve (reviewed in Kaewkhaw et al. 2012). This stage requires culture from 7 to 14 days but has been found to produce inconsistent results. Schwann cells are typically cultured in vitro in a medium rich in mitogens to stimulate cell proliferation, which in parallel stimulates fibroblast growth, and consequently is a contaminating factor if pure Schwann cultures are required. Historically, several methods have been developed to overcome this issue, such as isolating cells from neonatal or embryonic nerve tissue. Fibroblast numbers are reported to be low, and the extracellular matrix is immature and not fully developed. Another approach is separation of Schwann cells from fibroblasts or fibroblasts from Schwann cells using antimitotic chemicals (e.g., cytosine arabinoside), antibodies (anti-Thy-1), and complement-mediated cell lysis or crude physical techniques (pipette jetting). However, these are time-consuming, expensive, and technically demanding, and irrespective of the method chosen, the number and yield of Schwann cells obtained are typically low (reviewed in Kaewkhaw et al. 2012).

Haastert et al. reported on the development of a more selective method to obtain pure Schwann cells. This included the use of melanocyte growth medium containing forskolin, fibroblast growth factor-2, pituitary extract, and heregulin, which stimulates Schwann cell proliferation and minimizes fibroblast growth (Haastert et al. 2007). However, it was Kaewkhaw et al. who developed an efficient protocol to obtain pure Schwann cells from adult nerve tissue, with increased purity and a reduced time in culture, based on the substitution of the essential amino acid L-valine with D-valine in the culture medium. The concept of the method was the discovery that Schwann cells metabolize D-valine preferentially over fibroblasts (when isolated from the same nerve) due to differential expression of D-amino acid oxidase (DAAO) in the Schwann cells. These differences were identified on a mRNA and protein level in both Schwann and fibroblasts. Compared to Schwann cells, fibroblasts taken from the same nerve expressed DAAO at very low levels. The discovery permitted a breakthrough for isolating and culturing highly pure Schwann cells directly from

fresh adult nerve. Average Schwann cell purities of 97% are reported after 19 days without pre-degeneration, purification, or antimitotic steps (Kaewkhaw et al. 2012). Figure 3 shows a diagram of the steps of this protocol.

In addition, Schwann cells can be obtained from stem cells. Kaewkhaw et al. reported on the differentiation of Schwann cells taken from adipose-derived stem cells. Three different anatomical sites of subcutaneous, perinephric, and epididymal adipose tissue were sourced to harvest the cells. It was described that the source of adipose stem cells correlated with Schwann cell phenotype, glial antigen expression profile, and the capacity to encourage neuronal cell differentiation. Moreover, the presence of high levels of brain-derived neurotrophic factor and nerve growth factor and low levels of neurotrophin-3 in subcutaneous and perinephric fat was shown to be more effective than the Schwann cells obtained from epididymal fat (Kaewkhaw et al. 2011).

A deeper understanding of mechanotransduction pathways and mechanosensitive ion channels in Schwann cells has inspired approaches that include physical stimulation incorporated into isolation and cultivation procedures. Tsuang et al. discovered that low-intensity pulsed ultrasound (frequency, 1 MHz; duration, 2 min; duty cycle, 20%; total treatment time, 3 min) promotes rat Schwann cell proliferation and prevents cell death (Tsuang et al. 2011). Schuh et al. utilized extracorporeal shockwave treatment (ESWT) during the isolation process and found that ESWT increases cell yield, purity, and reactivity to pro-myelination stimuli. Furthermore, ESWT has been proven as an effective method to counteract Schwann cell senescence in prolonged culture (after 105 days) (Schuh et al. 2016). Similar effects regarding increased proliferation and reactivity to pro-myelination stimuli were found by Jung et al. using optogenetic stimulation (OS). OS of 473 nm with blue LEDs elevated intracellular calcium levels, leading to the effects described above (Jung et al. 2019).

One factor less in the spotlight concerning Schwann cell culture is their strong response to substrate stiffness. In the healthy, uninjured nerve presenting all structures (axons, myelinating Schwann cells, ECM), elasticity is around 50 kPa, while tissue culture polypropylene's (TCPP) elasticity is around 100,000 kPa (Gu et al. 2012; Skardal et al. 2013). Typical Schwann cell morphology (bipolar, elongated) has been shown to be a response to stiff surfaces, such as TCPP. Gu et al. demonstrated that the ideal substrate stiffness should be around 7.45 kPa to study Schwann cell biological functions (Gu et al. 2012). Evans et al. demonstrated that the response of Schwann cells to substrate stiffness can be utilized to modulate migration: in an in vitro durotaxis model, Schwann cells displayed lower migration on mono-stiffness surfaces compared to stiffness gradients (Evans et al. 2018).

As mentioned throughout this chapter, axonal outgrowth depends on the migration of Schwann cells within an environment (Bell and Haycock 2012). For this reason, in vitro models and applications are being studied to evaluate Schwann cell proliferation, migration, and morphology. Schwann cell cultures are essential to understand their role during nerve injury; however, it is necessary to understand their potential as well as their limitations to successfully link in vitro basic research to clinical application.

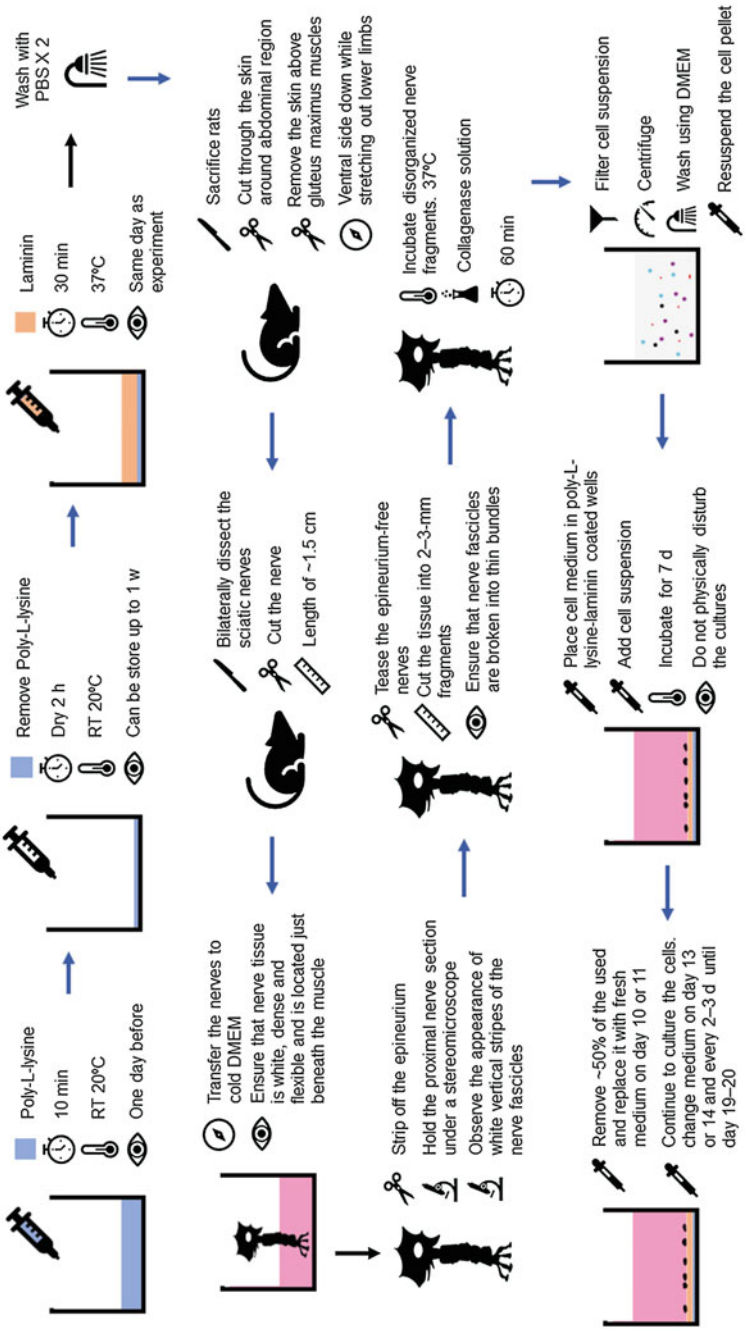


Fig. 3 Overview showing a selective protocol to isolate and purify Schwann cells from adult nerve (Kaewkhaw et al.)

5 Functional Assays for Schwann Cell Testing

5.1 Myelination Assays

The mechanisms associated with myelination and basal lamina formation in peripheral nervous system have been extensively studied, thanks to the development of in vitro co-cultures of Schwann cells with primary sensory neurons (Wood 1976; Carey and Bunge 1981; Eldridge et al. 1987). Myelination assays act as models for several myelin disorders, such as demyelination or delayed myelination. The principle underlying these assays is the cultivation of primary neurons on a layer of Schwann cells, stimulating neurite outgrowth and subsequently myelination (Hyung et al. 2015). Dorsal root ganglion (DRG) neurons are normally obtained from embryonic mouse (E13.5) or rat (E16), as a dissociated culture or in the form of organotypic explant. Other sources of primary neurons include retinal ganglion cells (Bähr et al. 1991) and motor neurons (Hyung et al. 2015).

Pioneering work carried out by Patrick Wood's group (Wood 1976; Carey and Bunge 1981; Eldridge et al. 1987) have shown that Matrigel, type I collagen, laminin, and poly-lysine (L and D) (Taveggia and Bolino 2018; Hyung et al. 2015) are important for myelination. The use of vitamins and growth factors is also important. Preliminary work established that Schwann cell-neuron co-cultures require human placental serum and chick embryo extract for myelin formation, which was enhanced by addition of ascorbic acid (Eldridge et al. 1987). Current protocols can be variable in the use of growth factors and supplements (fetal bovine serum, human placental serum, N2), but ascorbic acid and NGF seem to be necessary in all protocols to induce myelination and support neuronal growth, respectively (Taveggia and Bolino 2018; Eldridge et al. 1987; Hyung et al. 2015; He et al. 2010; Kumar et al. 2016). Newly formed myelin can be detected 1 week after the start of induction by several techniques, such as immunofluorescence, Western blotting, and transmission electron microscopy. The main markers of myelination include myelin proteins as, e.g., MBP, myelin protein zero, myelin-associated glycoprotein, PMP22, and galactosylceramide. Transcription factors associated with myelination include Sox10 and Krox20 (Taveggia and Bolino 2018; Hyung et al. 2015; He et al. 2010; Kumar et al. 2016; Wood et al. 1990; Honkanen et al. 2007).

6 In Vitro Models of Schwann Cells for the Treatment of Peripheral Nerve Injuries

Research has been conducted across a range of diverse applications for the treatment of peripheral nerve injuries. This includes use of Schwann cell cultures, as essential for stimulating axonal outgrowth. One of the most interesting characteristics of Schwann cells is their ability migrate and proliferate to form highly directional bands of Büngner, acting as guiding structures for axonal outgrowth. This ability to align has been found to be reproducible in several in vitro studies, for example, Ahmed and Brown using fibronectin fibers and neonatal rat Schwann cells, as well as

Dubey et al. using magnetically aligned collagen in a neonatal Schwann and dorsal root ganglion co-culture (Ahmed and Brown 1999; Dubey et al. 1999). Aside from natural materials, 3D in vitro peripheral nerve models have also been developed to analyze neurite formation and Schwann cell proliferation on synthetic scaffolds, e.g., aligned electrospun polycaprolactone (PCL) fibers. Results showed that when Schwann cells are co-cultured with neuronal cells, neurite formation is enhanced. Moreover, Schwann cells migrate along PCL fibers (Daud et al. 2012), and hence longer axon outgrowth is possible. Photopolymerizable polylactic acid (PLA) scaffolds can also be used to fabricate and evaluate Schwann cell growth, with results supporting that Schwann cells proliferate on similar synthetic scaffold materials (Koroleva et al. 2012).

Additionally, Schwann cells can be cultured as part of the dorsal root ganglion explants (DRGs), obtained from either rat or chick embryos. DRGs can be grown artificially in experimental nerve guide conduits (NGC) fabricated from a photocurable poly(ethylene glycol) (PEG) polymer by additive layer microstereolithography (μSL). Results showed that Schwann cells can migrate up to 9.5 mm from the DRG body after 14 days of culture in vitro. Axonal outgrowth and Schwann cell migration in such constructs are optimally visualized and quantified by lightsheet microscopy. Combining the technologies of PCL electrospun fibers and photocurable PEG NGCs, Behbehani et al. developed a 3D in vitro model to study Schwann cells and axon outgrowth from DRGs. In this study, PCL electrospun fibers were introduced inside an experimental NGC as a guidance scaffold. Isolated whole DRGs are placed on top of the fibers and cultured for up to 21 days. Results show that Schwann cells can migrate an average of 2.2 mm along fibers with average axonal outgrowth of 2.1 mm after 7 days and maximum axon lengths of >5 mm per construct observed after 21 days. These studies have two immediate areas of application: (i) in the study of complex 3D in vitro models to facilitate a range of underpinning research for neuronal-glial interactions (e.g., pharmacological studies or disease models) and (ii) for the analysis of biomedical nerve implants prior to in vivo evaluation. Initial data using the model herein interestingly supports Schwann cells in having a leading migratory role, possibly serving as guidance to regenerating axons via the deposition of extracellular matrix proteins (Behbehani et al. 2018).

Scaffolds fabricated with natural and synthetic materials are also functionalized with different molecules to stimulate Schwann cell differentiation and proliferation to aid nerve repair. Molecules used to impart biological activity to materials include growth factors; ECM proteins, such as laminin and fibronectin; and chemical functional groups. Silicone tubes functionalized with laminin, fibronectin, and fibronectin plus laminin all increase Schwann cell infiltration and migration (Bailey et al. 1993). Laminin has also been cross-linked to PCL-chitosan scaffolds, encouraging Schwann cell attachment and stimulating proliferation significantly in comparison to PCL-chitosan scaffolds with adsorbed laminin (Radoslaw et al. 2013). Moreover, PCL fibers were functionalized with neuregulin-1 (NRG1) to stimulate Schwann cell adhesion, proliferation, and directional growth. Results show positive outcomes as fibers guide the growing Schwann cells. NGR1, an important trophic factor for Schwann cells, encourages proliferation and enlargement (Tonazzini et al. 2017).

Lee and Schmidt fabricated an amine-functionalized polypyrrole to improve Schwann cell adhesion. Polypyrrole is an electrically conducting polymer with the capacity to modify the biological microenvironment through electricity. When Schwann cells were cultured on electrically stimulated polypyrrole, they show an increased secretion of brain-derived neurotrophic factor and nerve growth factor. Hence, if the surface was functionalized to improve Schwann cell adhesion, it could increase the production of these neurotrophins to enhance nerve repair (Lee and Schmidt 2015). Additionally, amine-functionalized nanodiamond is reported to encourage the growth of Schwann cells with an appropriate phenotype (Hopper et al. 2014). Polyurethane functionalized with poly(glycerol sebacate) (PGS) and aniline pentamer significantly improved Schwann cell myelin expression and sustained neurotrophin expression of nerve growth factor, brain-derived neurotrophic factor, and ciliary neurotrophic factor (CNTF) (Wu et al. 2016). CNTF is of major importance as it improves remyelination of regenerative nerves (Dinis et al. 2015).

In contrast, Arg-Gly-Asp (RGD) motifs have been added to functionalized PCL. This approach is reported to improve Schwann cell adhesion and proliferation, suggesting that Schwann cells spontaneously formed columns resembling bands of Büngner (Pérez et al. 2013). Gold nanoparticles functionalized with neuronal cell adhesion molecule L1 also stimulate Schwann cell process formation and proliferation (Schulz et al. 2013). Masand et al. functionalized a collagen hydrogels with polysialic acid (glycan) which improved Schwann cell proliferation as well as process extension (Masand et al. 2012). Electrospun scaffolds of poly(3-hydroxybutyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydeoxyvalerate) (PHBV) have also been functionalized with collagen I, H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS), H-Try-Ile-Gly-Ser-Arg-NH2 (YIGSR), or H-Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile-OH (p20) peptides which are reported to recreate the native ECM motifs for nerve regeneration. Schwann cells aligned along fibers, and the biofunctionalized scaffolds, supporting differentiation along the fibers (Masaeli et al. 2014).

In summary, various in vitro studies have been conducted on Schwann cell growth and migration, using functionalized materials that resemble the native microenvironment of nerve. As mentioned above, it is important to understand how Schwann cells proliferate and migrate in vitro due to their crucial role during nerve regeneration. Furthermore, this increased understanding of Schwann cell behavior can be targeted toward in vivo studies, where the complexity of the native tissue gives valuable information on how to design nerve repair therapies using Schwann cells.

7 In Vivo Applications of Schwann Cells in Peripheral Nerve Regeneration

Since Schwann cells have been recognized as one of the driving forces in peripheral nerve regeneration, many attempts have been made to transplant them to defect sites. For this purpose, a number of preclinical models have been developed over the last

decades. Small rodents comprise the vast majority of preclinical models. The most popular model for peripheral nerve regeneration is the rat sciatic nerve defect model, with around 63% of all tissue engineering-related studies, followed by the mouse sciatic nerve and rat peroneal nerve (reviewed by Angius et al. (2012)). Gap sizes in the rat sciatic nerve defect model typically range between 6 and 10 mm. However, for long-term studies, gap sizes larger than 30 mm are not uncommon (Karimi et al. 2014). The popularity of the rat sciatic nerve defect model can be explained by its clinically relevant diameter (sciatic nerve diameter of 1–2 mm, similar to nerves in the human hand), easy and reproducible surgical procedures, as well as its availability. The mouse sciatic nerve defect model uses gap sizes between 3 and 5 mm (Goulart et al. 2014; Wang et al. 2017) and offers the advantage of readily available transgenic models and the possibility of studying large groups. Criticism on both, rat and mouse models, have been made regarding gap size as well as rapid axonal regeneration and subsequently poor translatability into the clinic (Kaplan et al. 2015; Ronchi et al. 2019). Rabbit models allow regeneration through clinically more relevant gap sizes (up to 6 cm), however lack functional assays to evaluate regenerative outcomes (Wang et al. 2011). Larger animal models (sheep, pig, monkey) allow studies of genuinely longer gaps as well as larger diameters, with the disadvantage of longer regeneration times, higher animal and housing costs, as well as ethical issues, especially with nonhuman primates (Wang et al. 2011). It is well-known that animal models are far from perfect. However, it is necessary to be aware of each model's limitations, not just to choose the correct model for the application but also to interpret the results accordingly.

Historically, the autologous nerve graft can be considered the first transplantation of Schwann cells. Within its nerve structure, it contains Schwann cells that subsequently undergo Wallerian degeneration, presenting proliferating Schwann cells in the so-called Büngner cell state. It is the gold standard in peripheral nerve regeneration; however, low success rates have driven research not only to improve the autograft itself but to develop new functionalized conduits to deliver Schwann cells to the defect site (Yang et al. 2011; Secer et al. 2008). In order to improve the autograft, it has been demonstrated by several groups in preclinical models that pre-degeneration of nerve graft has beneficial effects on the axonal outgrowth and subsequent myelination (Danielsen et al. 1994; Kerns et al. 1993). Another limiting factor of autografts is their low availability. Addressing this issue, decellularized nerve grafts, presenting nerve basement membrane and extracellular matrix components, without eliciting an immune response, have become increasingly popular (Hudson et al. 2004). While available in larger quantities, the absence of Schwann cells has been noted as negatively influencing regeneration. Recellularizing nerve allografts with autologous Schwann cells has been investigated by Jiang et al. in a 40 mm ulnar nerve defect rhesus monkey model, finding similar results compared to autologous nerve graft and improved axonal regeneration compared to the empty decellularized graft (Jiang et al. 2016). Another approach that has gained reasonable attention more recently aims to understand the mismatch between the sensory autografts in a motor defect site. Aside from structural differences influencing axonal regeneration and functional outcome (Moradzadeh et al. 2008), it has been shown in

several studies that motor and sensory Schwann cells differ in their receptors as well as secretion of neurotrophic factors (Chu et al. 2008; Höke et al. 2006; Jesuraj et al. 2012). Interestingly, the rat sciatic nerve defect model (the most common model for peripheral nerve regeneration) does not allow distinction between phenotypes due to a highly mixed motor/sensory nerve architecture. Hercher et al. analyzed in a comprehensive study the spatiotemporal behavior of homotopic and heterotopic grafts using a rat femoral nerve defect model. It was found that the gene expression of several factors such as GDNF, BDNF, TrkB, and Cadm3, among others differ throughout the 10-week observation period between phenotypically matched and mismatched grafts (Hercher et al. 2019).

Aside from understanding the limitations of autografts, the development of tissue-engineered nerve grafts containing Schwann cells has gained considerable attention. One of the first engineered Schwann cell containing grafts reported by Guenard et al. utilized Matrigel as a carrying structure, within an acrylonitrile-vinyl chloride copolymer tube. It was found that the presence of Schwann cells had a beneficial effect on axonal growth, but more interestingly, strain-mismatched Schwann cells solicited a strong immune response (Guenard et al. 1992). Over the years, a variety of scaffold materials have been tested to deliver Schwann cells, among which collagen, fibrin, PCL, PLGA, chitosan and silk fibroin, and others can be found (Schuh et al. 2018; Xie et al. 2014; Yuan et al. 2004; Hsu et al. 2005). One consistent factor determined as being essential for the success of Schwann cell transplantation is scaffold architecture. This means that the inner structures of tubes should ideally mimic nerve structures to align Schwann cells and direct axonal outgrowth. One approach to achieve bio-mimicry was by Georgiou et al., designing a self-aligning neural tissue based on collagen and Schwann cells, supporting nerve regeneration in a 15 mm rat sciatic nerve defect model (Georgiou et al. 2013). Through addition of fibrin (a known Schwann cell proliferation enhancer) to the collagen-Schwann cell blend, axonal regeneration was further improved (Schuh et al. 2018). Another approach designed by Xie et al. was based on electrospinning poly(ε-caprolactone) into a conduit containing aligned fibers and seeded with Schwann cells prior to implantation into a 14 mm rat sciatic nerve defect. Results demonstrated less axonal regeneration compared to the autograft but improved compared with an empty tube (Xie et al. 2014). Owens et al. designed a fully cellular nerve graft, containing bone marrow mesenchymal stem cells and Schwann cells, assembled by bioprinting into a multi-luminar nerve graft, finding results comparable to the autologous nerve graft (Owens et al. 2013).

Several approaches, independent of the fabrication and material, have demonstrated that the presence of Schwann cells before implantation has a beneficial effect on the outcome. One of the recurrent questions and challenges in Schwann cell transplantation is survival after implantation. While in vitro parameters such as medium composition, nutrient, and oxygen supply can be controlled and monitored, this has proven difficult for in vivo transplanted grafts. First attempts to follow Schwann cell survival in vivo were made in the early 2000s, where Schwann cells stably expressing GFP were transplanted into a defect site. Grafts were found to contain GFP Schwann cells integrated into nerve regeneration processes 2 weeks

after implantation (Tohill et al. 2004). Gambhir et al. suggested a refined approach, transducing Schwann cells using a lentiviral vector with a nuclear localization signal fused with mCherry to improve differentiation between single cells for more precise cell counts (Gambhir et al. 2016). Long-term live monitoring was also achieved by Kimura et al, using *firefly*-luciferase-labeled neural crest-like cells (Kimura et al. 2018). However, all these approaches test the survival of transplanted cells in an existing conduit. In contrast, Coy et al. presented an interesting new approach to simulate axonal behavior within a nerve conduit using mathematical modeling, giving perspectives on integrating similar algorithms into prediction of vascularization and subsequently cell survival within nerve grafts (Coy et al. 2018).

Since survival of transplanted Schwann cell cannot be assured, an upcoming hot topic of the last decade has become more and more interesting in supporting and replacing cell therapy: exosomes and microvesicles have been proven to be potent helpers in regeneration of several tissues (e.g., cardiovascular or bone and muscle, reviewed by Sun et al. (2018)). Lopez-Verrilli et al. discovered that exosome-derived Schwann cells (in the Büngner cell state) promote axonal regeneration in a rat sciatic nerve defect model by modifying the morphology of the growth cone toward a regenerative phenotype. One of the factors identified was GTPase RhoA, known to play a role in growth cone collapse and axon retraction (Lopez-Verrilli et al. 2013).

8 Alternatives to Native Schwann Cells

With the advances of cell therapy and tissue engineering, the demand for primary cells has risen. As above, Schwann cells have historically been known as difficult to isolate, since there is no readily accessible source. Furthermore, as highlighted by Wakao et al, collecting Schwann cells is inevitably damaging a nerve. Several alternative sources have been proposed, including mesenchymal stem cells, embryonic stem cells, as well as induced pluripotent stem cells (Dezawa et al. 2001; Liu et al. 2012). Recently, mesenchymal stem cells (MSCs) have gained considerable interest as an alternative to the use of native Schwann cells. MSCs have been found in several tissues including the bone marrow, umbilical cord, adipose tissue, and amniotic membrane, among others (reviewed by Hass et al. (2011)). Aside from evident advantages such as a potential to differentiate into a several mesenchymal cell types (e.g., adipocytes, osteocytes, chondrocytes, and fibroblasts (Fan et al. 2008; Väänänen 2005; Dominici et al. 2006; Strem et al. 2005)), their immunogenic phenotype lacks major histocompatibility complex class II (MHC II, hindering T-cell stimulation and subsequently immunogenic responses (Javazon et al. 2004; Barry and Murphy 2004; Chamberlain et al. 2007)).

The first group to propose generating Schwann cell-like cells from MSCs was Dezawa et al., utilizing bone marrow-derived MSCs and a three-step protocol. Cells were primed toward a neural lineage with β-mercaptoethanol and retinoic acid, followed by differentiation into Schwann cell-like cells with bFGF, PDGF, heregulin, and forskolin (Dezawa et al. 2001). Several subsequent studies demonstrated robust differentiation from a variety of MSC sources, including the adipose

tissue, umbilical cord, and amniotic membrane (Wakao et al. 2014; Chen et al. 2019). Schwann-like cells have been found to express genes, transcription factors, and proteins associated with Schwann cells, including S100ß, p75NGFR, GFAP, SOX10, Krox20, and c-jun (Kaewkhaw et al. 2011; Wakao et al. 2014; Chen et al. 2019). Furthermore, they have also been shown to express myelin and stimulate neurite outgrowth in vitro *(*Xu et al. 2008*).* The latter was partially associated with exosome and RNA transfer, as an important component of the secretome (Ching et al. 2018). In preclinical studies, the performance of Schwann-like cells was evaluated as being comparable to native Schwann cells (e.g., when seeded into decellularized nerve grafts or chitosan conduits) (Fan et al. 2014; Ao et al. 2011; Wang et al. 2012). Given the availability of Schwann-like cells differentiated from human waste material such as the adipose tissue or amniotic membrane, those cells provide a solid alternative for in vitro testing of, e.g., pharmaceuticals or potential nerve conduits, as well as for implantation to augment peripheral nerve regeneration.

9 Conclusions

The above review highlights the essential role of Schwann cells in nerve development and function and their role in regeneration following injury. A range of studies have been critiqued that have revealed our understanding of Schwann cell behavior and how they interact to axons to encourage neurite outgrowth. Research has been conducted across a wide range of in vitro and in vivo studies reporting on the use of different biomaterials and models to elucidate nerve repair therapies that either use Schwann cells as a potential therapy or identify a central role in regeneration. Furthermore, isolation protocols are discussed that have been developed to obtain purified Schwann cell cultures that overcome previous technical limitations for Schwann cell therapy. In addition, a range of alternatives to primary Schwann sources are critiqued as alternatives to this.

References

Ahmed Z, Brown RA (1999) Adhesion, alignment, and migration of cultured Schwann cells on ultrathin fibronectin fibres. Cell Motil Cytoskeleton 42:331–343

Angius D, Wang H, Spinner RJ, Gutierrez-Cotto Y, Yaszemski MJ, Windebank AJ (2012) A systematic review of animal models used to study nerve regeneration in tissue-engineered scaffolds. Biomaterials 33(32):8034–8039

Ao Q et al (2011) The regeneration of transected sciatic nerves of adult rats using chitosan nerve conduits seeded with bone marrow stromal cell-derived Schwann cells. Biomaterials 32:787–796

Arancibia-Carcamo D, Attwell IL (2014) The node of Ranvier in CNS pathology. Acta Neuropathol 128(2):161–175

Bähr M, Hopkins JM, Bunge RP (1991) In vitro myelination of regenerating adult rat retinal ganglion cell axons by schwann cells. Glia 4(5):529–533

Bailey SB, Eichler ME, Villadiego A, Rich KM (1993) The influence of fibronectin and laminin during Schwann cell migration and peripheral nerve regeneration through silicon chambers. J Neurocytol 22(3):176–184

Balakrishnan A, Stykel MG, Touahri Y, Stratton JA, Biernaskie J, Schuurmans C (2016) Temporal analysis of gene expression in the murine Schwann cell lineage and the acutely injured postnatal nerve. PLoS One 11(4):1–32

Bandtlow CE, Heumann R, Schwab ME, Thoenen H (1987) Cellular localization of nerve growth factor synthesis by in situ hybridization. EMBO J 6:891–899

Barry FP, Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 36(4):568–584

Behbehani M, Glen A, Taylor CS, Schuhmacher A, Claeyssens F, Haycock JW (2018) Pre-clinical evaluation of advanced nerve guide conduits using a novel 3D in vitro testing model. Int J Bioprinting 4(1):1–12

Bell JHA, Haycock JW (2012) Next generation nerve guides: materials, fabrication, growth factors, and cell delivery. Tissue Eng Part B Rev 18(2):116–128

Bhatheja K, Field J (2006) Schwann cells: origins and role in axonal maintenance and regeneration. Int J Biochem Cell Biol 38(12):1995–1999

Blanchard AD et al (1996) Oct-6 (SCIP/Tst-1) is expressed in Schwann cell precursors, embryonic Schwann cells, and postnatal myelinating Schwann cells: comparison with Oct- 1, Krox-20, and Pax-3. J Neurosci Res 46(5):630–640

Bremer M et al (2011) Sox10 is required for Schwann-cell homeostasis and myelin maintenance in the adult peripheral nerve. Glia 59(7):1022–1032

Britsch S et al (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev 15:66–78

Bunge R (1986) Linkage between axonal ensheathment and basal lamina production by Schwann cells. Annu Rev Neurosci 9:305–328

Carey DJ, Bunge RP (1981) Factors influencing the release of proteins by cultured Schwann cells. J Cell Biol 91(3):666–672

Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 25(11):2739–2749

Chen HSM, Holmes N, Liu J, Tetzlaff W, Kozlowski P (2017) Validating myelin water imaging with transmission electron microscopy in a rat spinal cord injury model. NeuroImage 153:122–130

Chen W, Xiao S, Wei Z, Deng C, Nie K, Wang D (2019) Schwann cell-like cells derived from human amniotic mesenchymal stem cells promote peripheral nerve regeneration through a MicroRNA-214/c-Jun pathway. Stem Cells Int 2019:1–13

Chernousov MA, Yu WM, Chen ZL, Carey DJ, Strickland S (2008) Regulation of Schwann cell function by the extracellular matrix. Glia 56(14):1498–1507

Ching RC, Wiberg M, Kingham PJ (2018) Schwann cell-like differentiated adipose stem cells promote neurite outgrowth via secreted exosomes and RNA transfer. Stem Cell Res Ther 14:124–131

Chiono V, Tonda-Turo C (2015) Trends in the design of nerve guidance channels in peripheral nerve tissue engineering. Prog Neurobiol 131:87–104

Chu TH, Du Y, Wu W (2008) Motor nerve graft is better than sensory nerve graft for survival and regeneration of motoneurons after spinal root avulsion in adult rats. Exp Neurol 212:562

Couve E, Lovera M, Suzuki K, Schmachtenberg O (2018) Schwann cell phenotype changes in aging human dental pulp. J Dent Res 97(3):347–355

Coy RH, Evans OR, Phillips JB, Shipley RJ (2018) An integrated theoretical-experimental approach to accelerate translational tissue engineering. J Tissue Eng Regen Med 12(1):e53–e59

Cravioto H (1965) The role of Schwann cells in the development of human peripheral nerves. J Ultrastruct Res 12(5–6):634–651

Danielsen N, Kerns JM, Holmquist B, Zhao Q, Lundborg G, Kanje M (1994) Pre-degenerated nerve grafts enhance regeneration by shortening the initial delay period. Brain Res 666(2):250–254

Daud MFB, Pawar KC, Claeyssens F, Ryan AJ, Haycock JW (2012) An aligned 3D neuronal-glial co-culture model for peripheral nerve studies. Biomaterials 33(25):5901–5913

Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H (2001) Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. Eur J Neurosci 14(11):1771–1776

Dinis TM et al (2015) 3D multi-channel bi-functionalized silk electrospun conduits for peripheral nerve regeneration. J Mech Behav Biomed Mater 41:43–55
Doddrell RDS, Dun XP, Moate RM, Jessen KR, Mirsky R, Parkinson DB (2012) Regulation of Schwann cell differentiation and proliferation by the Pax-3 transcription factor. Glia 60(9):1269–1278
Doetsch F (2003) The glial identity of neural stem cells. Nat Neurosci 6(11):1127–1134
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
Dubey N, Letourneau PC, Tranquillo RT (1999) Guided neurite elongation and Schwann cell invasion into magnetically aligned collagen in simulated peripheral nerve regeneration. Exp Neurol 158(2):338–350
Dupin E, Real C, Glavieux-Pardanaud C, Vaigot P, Le Douarin NM (2003) Reversal of developmental restrictions in neural crest lineages: transition from Schwann cells to glial-melanocytic precursors in vitro. Proc Natl Acad Sci U S A 100:5229–5233
Eldridge CF, Bunge MB, Bunge RP, Wood PM (1987) Differentiation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal lamina assembly and myelin formation. J Cell Biol 105(2):1023–1034
Evans EB, Brady SW, Tripathi A, Hoffman-Kim D (2018) Schwann cell durotaxis can be guided by physiologically relevant stiffness gradients. Biomater Res 22:14
Fan H, Liu H, Toh SL, Goh JCH (2008) Enhanced differentiation of mesenchymal stem cells co-cultured with ligament fibroblasts on gelatin/silk fibroin hybrid scaffold. Biomaterials 29(8):1017–1027
Fan L, Yu Z, Li J, Dang X, Wang K (2014) Schwann-like cells seeded in acellular nerve grafts improve nerve regeneration. BMC Musculoskelet Disord 15:165
Frostick SP, Yin Q, Kemp GJ (1998) Schwann cells, neurotrophic factors, and peripheral nerve regeneration. Microsurgery 18(7):397–405
Gambhir HS et al (2016) Improved method to track and precisely count Schwann cells post-transplantation in a peripheral nerve injury model. J Neurosci Methods 273:86–95
Gao X, Daugherty RL, Tourtellotte WG (2007) Regulation of low affinity neurotrophin receptor (p75NTR) by early growth response (Egr) transcriptional regulators. Mol Cell Neurosci 36:501–514
Georgiou M, Bunting SCJ, Davies HA, Loughlin AJ, Golding JP, Phillips JB (2013) Engineered neural tissue for peripheral nerve repair. Biomaterials 34(30):7335–7343
Goulart CO et al (2014) A combination of Schwann-cell grafts and aerobic exercise enhances sciatic nerve regeneration. PLoS One 9(10):e110090
Gu Y et al (2012) The influence of substrate stiffness on the behavior and functions of Schwann cells in culture. Biomaterials 33(28):6672–6681
Guenard V, Kleitman N, Morrissey TK, Bunge RP, Aebischer P (1992) Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. J Neurosci 12(9):3310–3320
Haastert K, Mauritz C, Chaturvedi S, Grothe C (2007) Human and rat adult Schwann cell cultures: fast and efficient enrichment and highly effective non-viral transfection protocol. Nat Protoc 2:99
Hadlock JP, Sundback T, Hunter C, Cheney D, Vacanti M (2000) A polymer foam conduit seeded with Schwann cells promotes guided peripheral nerve regeneration. Tissue Eng 6(2):119–127
Hass R, Kasper C, Böhm S, Jacobs R (2011) Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. Cell Commun Signal 9(1):12
He Y et al (2010) Yy1 as a molecular link between neuregulin and transcriptional modulation of peripheral myelination. Nat Neurosci 13(12):1472–1480
Hercher D et al (2019) Spatiotemporal differences in gene expression between motor and sensory autografts and their effect on femoral nerve regeneration in the rat. Front Cell Neurosci 13:182
Höke A et al (2006) Schwann cells express motor and sensory phenotypes that regulate axon regeneration. J Neurosci 26(38):9646–9655

Honkanen H et al (2007) Isolation, purification and expansion of myelination-competent, neonatal mouse Schwann cells. Eur J Neurosci 26(4):953–964
Hopper AP et al (2014) Amine functionalized nanodiamond promotes cellular adhesion, proliferation and neurite outgrowth. Biomed Mater 9(4):045009
Hsu SH, Chen CY, Lu PS, Lai CS, Chen CJ (2005) Oriented Schwann cell growth on microgrooved surfaces. Biotechnol Bioeng 92(5):579–588
Hudson TW et al (2004) Optimized acellular nerve graft is immunologically tolerated and supports regeneration. Tissue Eng 10:1641–1651
Hyung S, Yoon Lee B, Park JC, Kim J, Hur EM, Francis Suh JK (2015) Coculture of primary motor neurons and Schwann cells as a model for in vitro myelination. Sci Rep 5:15122
Ide C (1996) Peripheral nerve regeneration. Neurosci Res 25:101–121
Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32(5):414–425
Jessen KR, Mirsky R (2002) Signals that determine Schwann cell identity. J Anat 200(4):367–376
Jessen KR, Mirsky R (2005) The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci 6(9):671–682
Jessen KR, Mirsky R (2008) Negative regulation of myelination: relevance for development, injury, and demyelinating disease. Glia 56(14):1552–1565
Jessen KR, Mirsky R (2016) The repair Schwann cell and its function in regenerating nerves. J Physiol 594(13):3521–31
Jesuraj NJ et al (2012) Differential gene expression in motor and sensory Schwann cells in the rat femoral nerve. J Neurosci Res 90(1):96–104
Jiang X, Lim SH, Mao Hai-Quan HQ, Chew SY (2010) Current applications and future perspectives of artificial nerve conduits. Exp Neurol 223(1):86–101
Jiang CQ, Hu J, Xiang JP, Zhu JK, Liu XL, Luo P (2016) Tissue-engineered rhesus monkey nerve grafts for the repair of long ulnar nerve defects: similar outcomes to autologous nerve grafts. Neural Regen Res 11(11):1845–1850
Jung K, Park JH, Kim S-Y, Jeon NL, Cho S-R, Hyung S (2019) Optogenetic stimulation promotes Schwann cell proliferation, differentiation, and myelination in vitro. Sci Rep 9(1):3487
Kaewkhaw R, Scutt AM, Haycock JW (2011) Anatomical site influences the differentiation of adipose-derived stem cells for Schwann-cell phenotype and function. Glia 59(5):734–749
Kaewkhaw R, Scutt AM, Haycock JW (2012) Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue. Nat Protoc 7(11):1996–2004
Kahan I, Moscarello MA (1985) Identification of membrane-embedded domains of lipophilin from human myelin. Biochemistry 24(2):538–544
Kaplan HM, Mishra P, Kohn J (2015) The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. J Mater Sci Mater Med 26(8):226
Karimi M et al (2014) Rat sciatic nerve reconstruction across a 30 mm defect bridged by an oriented porous PHBV tube with Schwann cell as artificial nerve graft. ASAIO J 60(2):224–233
Kerns JM, Danielsen N, Holmquist B, Kanje M, Lundborg G (1993) The influence of predegeneration on regeneration through peripheral nerve grafts in the rat. Exp Neurol 122(1):28–36
Kimura H et al (2018) Stem cells purified from human induced pluripotent stem cell-derived neural crest-like cells promote peripheral nerve regeneration. Sci Rep 8(1):10071
Koehler PJ, Bruyn GW, Pearce JM (2000) Neurological eponyms. Oxford University Press, Oxford
Koroleva A, Gill AA, Ortega I, Haycock JW, Schlie S, Gittard SD, Chichkov BN, Claeyssens F (2012) Two-photon polymerization-generated and micromolding-replicated 3D scaffolds for peripheral neural tissue engineering applications. Biofabrication 4:025005
Kumar R et al (2016) Adult skin-derived precursor Schwann cells exhibit superior myelination and regeneration supportive properties compared to chronically denervated nerve-derived Schwann cells. Exp Neurol 278:127–142

Le N, Nagarajan R, Wang JYT, Araki T, Schmidt RE, Milbrandt J (2005) Analysis of congenital hypomyelinating Egr2Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell differentiation and myelination. Proc Natl Acad Sci U S A 102(7):2596–2601

Lee JY, Schmidt CE (2015) Amine-functionalized polypyrrole: inherently cell adhesive conducting polymer. J Biomed Mater Res Part A 103(6):2126–2132

Liu Q et al (2012) Human neural crest stem cells derived from human ESCs and induced pluripotent stem cells: induction, maintenance, and differentiation into functional Schwann cells. Stem Cells Transl Med 1:266–278

Lopez-Verrilli MA, Picou F, Court FA (2013) Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. Glia 61(11):1795–1806

Martini R, Schachner M (1997) Molecular bases of myelin formation as revealed by investigations on mice deficient in glial cell surface molecules. Glia 19(4):298–310

Masaeli E et al (2014) Peptide functionalized polyhydroxyalkanoate nanofibrous scaffolds enhance Schwann cells activity. Nanomed Nanotechnol Biol Med 10(7):1559–1569

Masand SN, Perron IJ, Schachner M, Shreiber DI (2012) Neural cell type-specific responses to glycomimetic functionalized collagen. Biomaterials 33(3):790–797

Masson P (1932) Experimental and spontaneous schwannomas (peripheral gliomas): II. Spontaneous schwannomas. Am J Pathol 8(4):389–416.11

Meier C, Parmantier E, Brennan A, Mirsky R, Jessen KR (1999) Developing schwann cells acquire the ability to survive without axons by establishing an autocrine circuit involving insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB. J Neurosci 19:3847

Menorca RMG, Fussell TS, Elfar JC (2013) Nerve physiology. Mechanisms of injury and recovery. Hand Clin 29:317–330

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119(1):45–54

Moradzadeh A et al (2008) The impact of motor and sensory nerve architecture on nerve regeneration. Exp Neurol 212(2):370–376

Murray-Dunning JW, McArthur C, Sun SL, McKean T, Ryan R, Haycock AJ (2011) Three-dimensional alignment of Schwann cells using hydrolysable microfiber scaffolds: strategies for peripheral nerve repair. In: Haycock JW (ed) 3D cell culture: methods and protocols, methods in molecular biology, vol 695. Springer Science+Business Media, pp 155–166

Owens CM, Marga F, Forgacs G, Heesch CM (2013) Biofabrication and testing of a fully cellular nerve graft. Biofabrication 5:045007

Painter MW et al (2014) Diminished Schwann cell repair responses underlie age-associated impaired axonal regeneration. Neuron 83(2):331–343

Parkinson DB et al (2008) c-Jun is a negative regulator of myelination. J Cell Biol 181(4): 625–637

Pérez ER, García Cruz DM, Araque Monrós MC, Gómez-Pinedo U, Pradas MM, Escobar Ivirico JL (2013) Polymer chains incorporating caprolactone and arginine-glycine-aspartic acid functionalities: synthesis, characterization and biological response in vitro of the Schwann cell. J Bioact Compat Polym 28(1):50–65

Poliak E, Peles S (2003) The local differentiation of myelinated axons at nodes of Ranvier. Natl Rev 4(12):968

Radoslaw X, Valmikinathan J, Kalyon CM, Yu DM (2013) Laminin functionalizes biomimetic nanofibres for nerve tissue engineering. J Biomater Tissue Eng 3(4):494–502

Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389:725–730

Roberts SL et al (2017) Sox2 expression in Schwann cells inhibits myelination in vivo and induces influx of macrophages to the nerve. Development 144(17):3114–3125

Ronchi G et al (2019) The median nerve injury model in pre-clinical research – a critical review on benefits and limitations. Front Cell Neurosci 13:288

Ryu EJ et al (2007) Misexpression of Pou3f1 results in peripheral nerve hypomyelination and axonal loss. J Neurosci 27(43):11552–11559
Saladin KS (2014) The nervous system I: nervous tissue. In: Human Anatomy. McGraw-Hill, New York, pp 349–368
Schuh CMAP et al (2016) Extracorporeal shockwave treatment: a novel tool to improve Schwann cell isolation and culture. Cytotherapy 18:760–770
Schuh CMAP, Day AGE, Redl H, Phillips J (2018) An optimized collagen-fibrin blend engineered neural tissue promotes peripheral nerve repair. Tissue Eng A 4(1):200–210
Schulz F et al (2013) Gold nanoparticles functionalized with a fragment of the neural cell adhesion molecule L1 stimulate L1-mediated functions. Nanoscale 5(21):10605–10617
Secer HI, Daneyemez M, Tehli O, Gonul E, Izci Y (2008) The clinical, electrophysiologic, and surgical characteristics of peripheral nerve injuries caused by gunshot wounds in adults: a 40-year experience. Surg Neurol 69:143
Sherman L, Stocker KM, Morrison R, Ciment G (1993) Basic fibroblast growth factor (bFGF) acts intracellularly to cause the transdifferentiation of avian neural crest-derived Schwann cell precursors into melanocytes. Development 118(4):1313–1326
Simons M, Trotter J (2007) Wrapping it up: the cell biology of myelination. Curr Opin Neurobiol 17(5):533–540
Skardal A, Mack D, Atala A, Soker S (2013) Substrate elasticity controls cell proliferation, surface marker expression and motile phenotype in amniotic fluid-derived stem cells. J Mech Behav Biomed Mater 17:307–316
Stampfli R (1954) Saltatory conduction in nerve. Physiol Rev 34(1):101–112
Stoll G, Jander S, Myers RR (2002) Degeneration and regeneration of the peripheral nervous system: from Augustus Waller's observations to neuroinflammation. J Peripher Nerv Syst 7:13–27
Stolt CC, Wegner M (2016) Schwann cells and their transcriptional network: evolution of key regulators of peripheral myelination. Brain Res 1641:101–110
Stratton JA et al (2017) Purification and characterization of schwann cells from adult human skin and nerve. eNeuro 4(3):1–15
Strem BM et al (2005) Multipotential differentiation of adipose tissue-derived stem cells. Keio J Med 54(3):132–141
Sun B, Peng J, Wang S, Liu X, Zhang K, Zhang Z, Wang C, Jing X, Zhou C, Wang Y (2018) Applications of stem cell-derived exosomes in tissue engineering and neurological diseases. Rev Neurosci 29:531
Taveggia C, Bolino A (2018) In: Woodhoo A (ed) DRG neuron/Schwann cells myelinating cocultures BT – myelin: methods and protocols. Springer New York, New York, pp 115–129
Taveggia C et al (2005) Neuregulin-1 type III determines the ensheathment fate of axons. Neuron 47(5):681–694
Tohill MP, Mann DJ, Mantovani CM, Wiberg M, Terenghi G (2004) Green fluorescent protein is a stable morphological marker for Schwann cell transplants in bioengineered nerve conduits. Tissue Eng 10(9–10):1359–1367
Tonazzini I, Moffa M, Pisignano D, Cecchini M (2017) Neuregulin 1 functionalization of organic fibers for Schwann cell guidance. Nanotechnology 28:155303
Tsuang Y-H et al (2011) Effects of low intensity pulsed ultrasound on rat Schwann cells metabolism. Artif Organs 35(4):373–383
Väänänen HK (2005) Mesenchymal stem cells. Ann Med 37(7):469–479
Wakao S, Matsuse D, Dezawa M (2014) Mesenchymal stem cells as a source of schwann cells: their anticipated use in peripheral nerve regeneration. Cells Tissues Organs 200:31–41
Wang PH, Tseng IL, Hsu SH (2011) Bioengineering approaches for guided peripheral. J Med Biol Eng 31(3):151–160
Wang Y et al (2012) Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. Neurosci Lett 514:96–101
Wang Y et al (2017) The effect of co-transplantation of nerve fibroblasts and Schwann cells on peripheral nerve repair. Int J Biol Sci 13(12):1507–1519

Wiley-Livingston CA, Ellisman MH (1980) Development of axonal membrane specializations defines nodes of Ranvier and precedes Schwann cell myelin elaboration. Dev Biol 79:334–355
Wood PM (1976) Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. Brain Res 115:361–375
Wood PM, Schachner M, Bunge RP (1990) Inhibition of schwann cell myelination in vitro by antibody to the L1 adhesion molecule. J Neurosci 10:3635–3645
Woodhoo A et al (2009) Notch controls embryonic Schwann cell differentiation, postnatal myelination and adult plasticity. Nat Neurosci 12(7):839–847
Wu Y, Wang L, Guo B, Shao Y, Ma PX (2016) Electroactive biodegradable polyurethane significantly enhanced Schwann cells myelin gene expression and neurotrophin secretion for peripheral nerve tissue engineering. Biomaterials 87:18–31
Xie J et al (2014) Nerve guidance conduits based on double-layered scaffolds of electrospun nanofibers for repairing the peripheral nervous system. ACS Appl Mater Interfaces 6(12):9472–9480
Xu Y et al (2008) Myelin-forming ability of Schwann cell-like cells induced from rat adipose-derived stem cells in vitro. Brain Res 1239:49–55
Yang M, Rawson JL, Zhang EW, Arnold PB, Lineaweaver W, Zhang F (2011) Comparisons of outcomes from repair of median nerve and ulnar nerve defect with nerve graft and tubulization: a meta-analysis. J Reconstr Microsurg 27(8):451–460
Yuan Y, Zhang P, Yang Y, Wang X, Gu X (2004) The interaction of Schwann cells with chitosan membranes and fibers in vitro. Biomaterials 25(18):4273–4278

Stem Cells: Umbilical Cord/Wharton's Jelly Derived

John T. Walker, Armand Keating, and John E. Davies

Contents

J. T. Walker
Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, ON, Canada
e-mail: jwalk29@uwo.ca

A. Keating
Institute of Biomaterials and Biomedical Engineering, Toronto, ON, Canada

University of Toronto, Toronto, ON, Canada

Cell Therapy Program, University Health Network, Toronto, Canada

Arthritis Program, Krembil Research Institute, University Health Network, Toronto, Canada

Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
e-mail: armand.keating@uhn.ca

J. E. Davies (✉)
Institute of Biomaterials and Biomedical Engineering, Toronto, ON, Canada

Faculty of Dentistry, Toronto, ON, Canada
e-mail: jed.davies@utoronto.ca

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_10

Abstract

As a commonly discarded tissue, the umbilical cord contains a rich source of mesenchymal stromal cells, which are therefore obtained non-invasively. As a perinatal population, replicative senescence is delayed and cell expansion is expedited, enabling collection of many clinically relevant doses from a single donor cord at low passage numbers. In this chapter, we will discuss the structure of the umbilical cord and the various stromal populations contained within that have been described. We also highlight the lack of consensus on both anatomical descriptors of the cord tissue, and standardized isolation techniques for these different populations, which together with insufficient methodological transparency may be hampering progress within the field. We then review the basic and preclinical models of disease that have been targets of umbilical cord-derived mesenchymal stromal cells. Finally, we close with a discussion of their use in clinical trials.

1 Introduction

The human umbilical cord is increasingly being employed as a tissue source of cells for cell therapy. While cord blood has been used therapeutically since 1988, the harvesting of cells from the structural tissue of the cord dates from the first isolation of human umbilical vein endothelial cells in 1963 (Maruyama 1963), although in all studies they have been limited to laboratory experiments, or clinically related assays, rather than therapeutic uses. More recently, since 2009, cell populations harvested from the nonvascular tissues of the umbilical cord have been employed for many different clinical targets. While the exact cell populations isolated from the cord are often not evident, and potentially include multiple unique subpopulations as discussed below, they are all generally described as MSCs.

Most authors now define an MSC by the minimal criteria suggested by the International Society for Cellular Therapy (ISCT) elaborated in their position paper of 2006 (Dominici et al. 2006). In the latter, the term mesenchymal stromal cell (MSC), rather mesenchymal stem cell, was proposed since evidence of the self-renewal and multi-lineage differentiation potential that define a stem cell were not generally provided by authors. We use the term MSC herein to describe the cell population derived from the connective tissue of the human umbilical cord, or Wharton's Jelly. But we would also point out that some authors have included the amniotic epithelium, the smooth muscle of the tunica media of the umbilical vessels, and even their endothelial linings in their harvested populations. Nevertheless, our focus herein will be on MSC populations harvested from the nonvascular tissue of

the human umbilical cord, the basic and preclinical studies that have been carried out both in vitro but predominantly in vivo in animal models, and the range of clinical studies that have been initiated using these cells.

However, we start by briefly reviewing the structure of the human umbilical cord, and the context of this tissue source in light of all MSC tissue sources being employed in clinical studies.

2 The Structure of the Human Umbilical Cord

At term, the human umbilical cord is approximately 60cms long with an average diameter of 1.5 cm. It has an outer covering of a single layer of amniotic epithelium and contains three vessels, a vein and 2 arteries, that are surrounded by a mucoid connective tissue called Wharton's Jelly (Wharton 1656). A single cross-section such as that in Fig. 1 illustrates the arrangement of these component parts. Importantly, in the human umbilical cord the vessels comprise only a tunica media and an endothelial lining. The role of the adventitia is borne by the Wharton's Jelly surrounding the vessels and known as the perivascular Wharton's Jelly. Distal to the perivascular jelly both cells and matrix become sparse, and clefts which contain only ground substance, are evident until the narrow cleft-free sub-amniotic zone immediately below the amniotic epithelium, which is commonly only one or two cells thick. We have recently described, elsewhere, the detailed anatomical structure of the human umbilical cord, its embryological derivation, together with some comparative anatomy for other commonly employed species (Davies et al. 2017). However, it is important to emphasize that until there is common agreement on

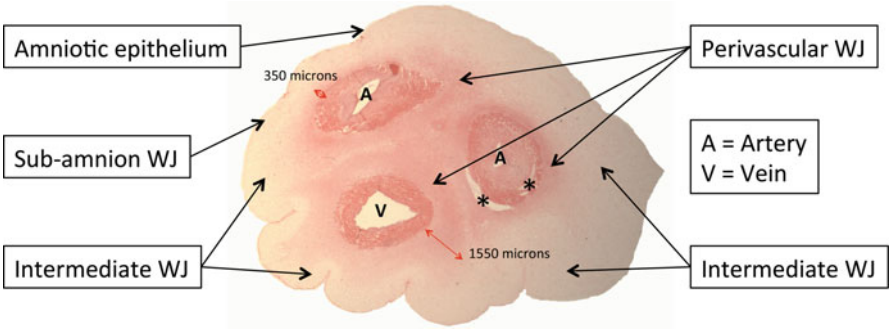


Fig. 1 A paraffin embedded cross-section of the human umbilical cord stained with hematoxylin and eosin. Shows an outer amniotic epithelium and three vessels contained within Wharton's Jelly (WJ), which extend from the tunica media of the vessels to the amniotic epithelium. Wharton's Jelly is denser in the perivascular zones, due to an increase in both cells and matrix, and measures 350–1550 microns deep in this sample (as marked). The paucity of staining in areas beyond the perivascular zones is, in part, due to the presence of clefts (visible when the image is enlarged) in the Intermediate WJ. The perivascular regions are separated by narrow regions of intervascular jelly that also contains clefts. The asterisks mark artifacts of preparation. Total Width = 15 mm

terminology used to describe either the anatomy of the cord or the cell populations harvested, it will be difficult to make detailed comparisons between the increasing numbers of studies employing this important tissue source.

Can and Karahuseyinoglu identified six zones within the human umbilical cord: (1) the surface (amniotic) epithelium, (2) subamniotic stroma, (3) clefts, (4) intervascular stroma, (5) perivascular stroma, and (6) the vessels (Can and Karahuseyinoglu 2007). They considered only zone 4 to be Wharton's Jelly, although most authors would describe Wharton's Jelly to comprise all the tissue outside the tunica media of the three vessels and bounded by the amniotic epithelium (zones 2–5 inclusive). Schugar et al., who described the perivascular zone as having an average depth of 430 microns, showed that 45% of the cells in Wharton's Jelly are found in zone 5 (Schugar et al. 2009). Of these anatomical structures of the cord, the descriptions of zones 1, 3, and 6 are generally agreed upon by the majority of authors. However, the description of zone 5 (and thus also zone 4) is highly variable between authors and ranges from a layer only 2 cells thick (Kita et al. 2010) or less than 500 microns deep (Troyer and Weiss 2008; Coskun and Can 2015) to 750–2000 microns deep (Subramanian et al. 2015) or 350–1550 microns as shown in Fig. 1. Reference to Fig. 1 will also make it clear that the depth of zone 4 is approximately 350 microns and that the 6 zone classification takes no account of the regions labeled Intermediate WJ in Fig. 1, which are quite extensive, although not rich in cells. Table 1 accompanying Fig. 1 provides a summary of the anatomical structure and classification of the human umbilical cord. From a cell harvesting perspective, it is relatively easy to isolate the perivascular tissue for cell extraction by numerous methods. Also, with fine dissection, it is possible to separate the Sub-Amnion, about 150 microns of Wharton's Jelly, from both the underlying Intermediate WJ and Perivascular WJ, and the overlying Amniotic epithelium. However, it would be

Table 1 The Anatomical Compartments of the Human Umbilical Cord. The Intervascular Wharton's Jelly (WJ) is not included here as an independent category, although it can be identified (see Fig. 1), because it cannot realistically be dissected from the Perivascular and Intermediate WJs, respectively. The endothelial cells of the tunica intima of the umbilical vein are known as human umbilical vein endothelial cells (HUVECs) while the Perivascular cells are known as human umbilical cord perivascular cells (HUCPVCs)

The Human Umbilical Cord					
Amnion	Wharton's Jelly			Vessels	
Epithelium	Sub-amnion WJ	Intermediate WJ	Perivascular WJ (intervascular WJ)	Tunica Media	Tunica Intima
1–3 cells thick	100–150 microns thick	Sparse matrix and cells of varying, irregular, dimensions	200–2000 microns thick	Two layers of smooth muscle orthogonally arranged	Single layer of endothelial cells
Cord "lining"		The WJ containing clefts	A functional adventia		

exceedingly difficult to isolate either the Intermediate WJ from both the Sub-Amnion WJ and the Perivascular WJ, or the Intervascular WJ (not marked in Fig. 1) from all other regions of the WJ.

These structural considerations are important since some authors have claimed to harvest cells from only selected zones of WJ, while others have reported that they extracted cells from Wharton's Jelly, without defining the exact tissue to which they are referring. We define Wharton's Jelly (see Table in Fig. 1) as all the mucoid tissue surrounding the vessels and extending to the underside of the enveloping amniotic epithelium. Furthermore, we define the perivascular tissue as that tissue, which is cell and matrix rich when compared to the remainder of Wharton's Jelly, and which surrounds the three vessels of the cord. This tissue has been previously referred to as the adventitia of the cord vessels (Nanaev et al. 1997). If the cord vessels are manually removed from the cord, after longitudinally opening or stripping the amniotic epithelium, the perivascular tissue remains adherent to the vessel wall as we, and others (Farias et al. 2011), have shown. However, we also caution that the arrangement of the vessels, their girth, and that of the cord itself, and thus the dimensions of the various zones mentioned are variable, although with appropriate staining, easily visualized.

3 Wharton's Jelly as a Source of MSCs

The human umbilical cord is a rich source of MSCs. Indeed, it is the advent of MSC biology that has driven the increasing interest in umbilical cord tissue as witnessed by the increasing number of publications in the last two decades (see Fig. 2). This is in part

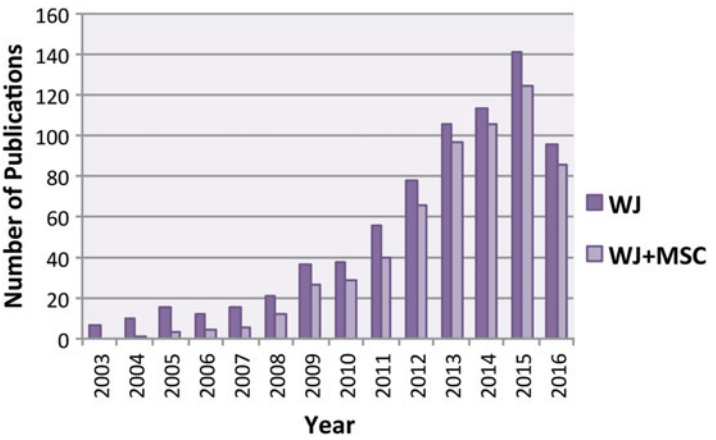


Fig. 2 A simple PubMed search using the term "Wharton's Jelly" returned seven publications in 2003 increasing to 141 in 2015. After 2007, the majority of papers also reference the term "MSC." (Note: 2016 for Jan–Oct only)

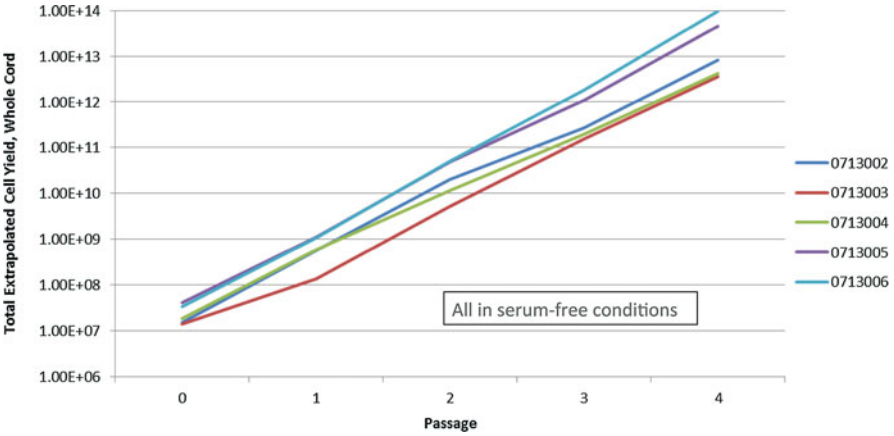


Fig. 3 Cell populations extracted from the perivascular tissue of the human umbilical cord can be rapidly expanded, at low passage, to vast numbers in serum-free conditions suitable for clinical use. (Data courtesy of Tissue Regeneration Therapeutics Inc.)

due to the high cell yields, high colony forming unit frequencies, and short population doubling times that have been reported by many authors as illustrated in Fig. 3.

The yield of MSC from Wharton's Jelly has been shown to depend upon the method of cell extraction employed. Using a CFU-F assay as a surrogate MSC measure, this can be illustrated by a comparison of Sarugaser et al. (2005) and Lu et al. (2006). While the former reports a CFU-F of 1:300 at harvest from an isolated UC perivascular population (Sarugaser et al. 2009), Lu et al. reported a CFU-F of 1:1609 (Lu et al. 2006), from cell populations harvested from minced whole cord, which is still considerably higher than the values of 1:10,000 to less than 1:100,000 reported for bone marrow MSCs (Caplan 2007).

Thus, it is not surprising that Wharton's Jelly has become an important contributory source of cells for MSC clinical trials as seen in Fig. 4. Wharton's Jelly cells contribute to the almost 50% of MSC clinical trials that employ allogeneic cells. To our knowledge, there has been no report of autologous use of these cells to date. The first four trials were in 2009, but there are currently 68 individual trials (including those in more than one trial phase) registered on ClinicalTrials.gov. This, therefore, represents a significant proportion of the most recently registered allogeneic MSC trials.

These trials have been preceded by a large number of basic and preclinical studies. The following, while not comprehensive, provides examples of the breadth of these studies. Can and Karahuseyinoglu provided an excellent review in 2007 and several others have been published since. However, we append here an additional section on those papers that have been published as outcomes of the clinical trials mentioned above employing Wharton's Jelly cells.

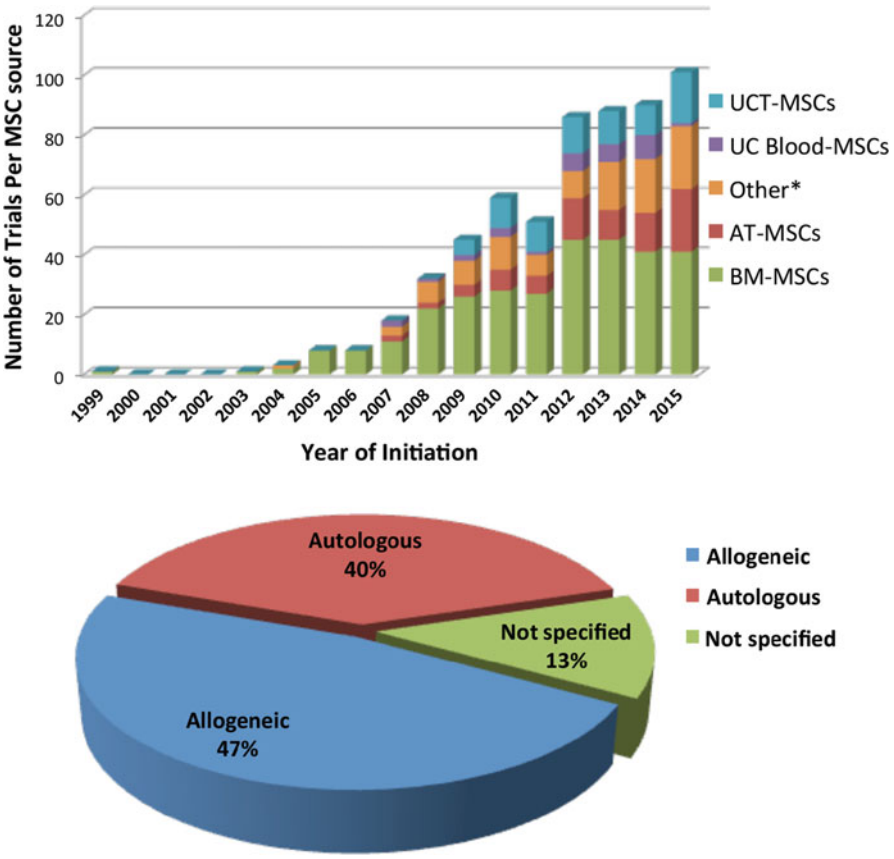


Fig. 4 Above: MSCs from multiple tissue sources have been employed in clinical trial to date. Below: Of these trials, 47% have used unmatched, allogeneic cells; 40% were autologous, while the remainder was not specified. (Data courtesy of Tissue Regeneration Therapeutics Inc. and obtained from ClinicalTrials.gov up to the end of 2014)

4 UC MSCs in Animal Models

While there is still little known about the development and origins of MSCs from the umbilical cord, their utility in therapeutics has been reported for a diverse range of maladies in animal models. Of critical importance, like all described MSC populations, UC-MSCs display an immune-privileged phenotype. Their responsive and adaptable anti-inflammatory, anti-apoptotic, and pro-angiogenic nature are often cited as sources for their wide-ranging utility. Additionally, their rapid ex vivo expansion and capacity for a high number of population doublings allows for

rapid harvest of clinically relevant doses as well as the ability to genetically modify these cells, turning them into biological factories which can be employed in situ. Here we will review several of the animal models in which umbilical cord MSCs have been studied.

Before reviewing these animal models, we consider it critical to emphasize the difficulty in making comparisons between studies when the methods of cell extraction are not provided in sufficient detail, or the zones of the cord from which cells are extracted are either not or differentially defined. To illustrate this, in their otherwise comprehensive paper, Subramanian et al. (2015) specifically chose to undertake a comparative characterization of cells derived from various compartments of the human umbilical cord. They compared cell populations from the amniotic membrane, the sub-amnion or "cord lining," the intervascular Wharton's Jelly, and the perivascular region. Histology was used to illustrate the various regions with the perivascular area labeled in similar manner to that in Fig. 1 herein. However, they provided no specific details of how they isolated cells from these different areas. Rather, they stated that the cells were isolated "using published established derivation protocols for each compartment" and list seven previously published papers for the methods. The latter are compared in Table 2. Within these references, one did not employ umbilical cord, while another was a review article that provided no specific cell derivation protocols; two papers diced whole cord tissue for explant cultures

Table 2 The papers referenced by Subramanian et al. (2015) to identify the method of isolation of cells from the various regions of the human umbilical cord

Ref # from Subramanian et al. 2015	Ref. in Bibliography	Method
3	Sarugaser et al. (2005)	Cord: Amnion opened, each vessel with surrounding WJ pulled away. Vessels tied, looped, WJ collagenase digested from vessels
4	Ilancheran et al. (2007)	Cord not used. Amnion separated from Chorion to isolate human amniotic epithelial cells (hAECs)
5	Troyer and Weiss (2008)	REVIEW: focuses on phenotype of UC-derived cells. Provides no cell derivation protocols. Note: Cartoon of perivascular region is limited to a thin surrounding of 1 artery and vein – thus different to Subramanian et al. (2015)
6	Schugar et al. (2009)	Cord: Whole pieces used either for (i) explant cultures or (ii) for enzymatic digestion in either dispase or collagenase
7	Kita et al. (2010)	Cord: Opened and WJ identified with phenol red, then scraped off while outer envelop provided sub-amnion. Note: perivascular region comprises a 2 cell thick layer
15	Fong et al. (2010)	Cord: Cut open and inner surface laid into collagenase/hyaluronidase solution without removing vessels. WJ subsequently scraped into fresh medium
18	Bosch et al. (2012)	Cord: Cut into small pieces and seeded as either single or multiple pieces for explant cultures

where every tissue in the cord would have been represented; two removed Wharton's Jelly by different means, neither of which distinguished between different regions of the cord and one specifically isolated cells from the perivascular jelly. It is thus impossible to determine how Subramanian et al. (ibid) identified the cells originating in the various regions of Wharton's Jelly. This lack of information is not uncommon in the studies quoted in the following sections.

4.1 Inflammatory Diseases

Several studies have shown that umbilical cord (UC)-MSCs share anti-inflammatory properties with their bone-marrow derived counterparts in vitro (Ennis et al. 2008; Payne et al. 2013; Donders et al. 2015). Consequently, UC-MSCs have been investigated in a range of diseases linked with excessive inflammation. Many of these diseases involve an improper balance between pro- and anti-inflammatory cells and their regulators. Ideally, a therapeutic could modify this imbalance to ameliorate tissue damage without significantly impairing the host response to invading pathogens. With the ability to respond and adapt to their environment, MSCs hold promise in these indications.

Autoreactivity of B and T cells with proteins of the myelin sheath has been suggested to be at the root of multiple sclerosis (MS) (McQualter and Bernard 2007; Steinman 2014). Experimental autoimmune encephalomyelitis (EAE) is an inducible animal model used to mimic this autoimmune reaction in MS. UC-MSCs have been explored in these models to limit the autoimmune destruction and improve regeneration of the myelin sheath. However, across three independent studies, the results have been variable. In a comparison of different MSC origins, Payne et al. showed that while bone marrow (BM)-MSCs display a greater anti-inflammatory response in vitro, only adipose derived (Ad)-MSCs and UC-MSCs significantly reduced the clinical score of mice compared to a PBS injected control (Payne et al. 2013). Still, there were no significant differences between any of the MSC groups, and improvements, which were only modest, could only be attained if cells were injected prior to the onset of symptoms (Payne et al. 2013). The authors linked the discrepancy between in vivo performance and in vitro anti-inflammatory response to differences in cell homing. Importantly, UC-MSCs and Ad-MSCs expressed a wider range of receptors involved in chemotaxis, as well as adhesion molecules used for extravasation at sites of injury when compared to BM-MSCs. Although UC-MSCs were not tested for homing capacity, they showed that Ad-MSCs could enter the CNS whereas BM-MSCs were unable to do so. Conversely, there were two studies showing minor improvements following UC-MSC administration, if injected following the onset of symptoms. Donders et al. found that the disease burden could be lessened, but only temporarily following UC-MSC injection whether injected at the onset of symptoms or even after a prolonged duration of symptoms (Donders et al. 2015). Notably, the improvements were not permanent and returned to levels of controls over time. In a study with the most promising results, Liu et al. showed lasting improvements in animals injected with UC-MSCs after the onset of disease

(Liu et al. 2013). These results were further supported with improved histological outcomes and even improved remyelination. These differences between studies, which may in part be due to differences in the disease model (Donders et al. 2015), emphasize a need for further exploration into the use of UC-MSCs for EAE. Of interest, in another approach to improve EAE progression, Agah et al. (2013) investigated treatment with oligodendrocyte progenitor cells derived from UC-MSC precursors (Agah et al. 2013). When these differentiated cells were injected prior to the onset of symptoms, clinical grade of EAE was improved to the end of the study and the extent of demyelination was reduced.

Crohn's disease and ulcerative colitis, together making up the inflammatory bowel diseases (IBD), involve excessive immune responses to inappropriate targets within the digestive tract including the resident gut flora (Bouma and Strober 2003). Although the etiology of IBD is unknown, animal models have been developed to mimic the inflammation within the digestive tract and have provided much insight into these diseases. Once again, chosen for their global anti-inflammatory properties, MSCs are a prime candidate for treating IBD. UC-MSCs have proven to be useful in ameliorating chemical-induced colitis in mice (Liang et al. 2011; Lin et al. 2015). In two independent studies, UC-MSCs were shown to home to sites of inflammation within the colon, reduce the inflammatory response, and decrease symptoms of colitis (Liang et al. 2011; Lin et al. 2015). In both cases, this was confirmed through histological analysis. Interestingly, another group has sought to improve upon the natural benefits of UC-MSCs for this indication by enhancing their immunosuppressive effects. Pretreatment of UC-MSCs with IL-1β (Fan et al. 2012) or genetic modification with an IFNγ expression construct (Chen et al. 2015) both improved treatment efficacy over unmodified UC-MSCs. Priming UC-MSCs with IL-1β led to improved homing as well as a modified immune response promoting a greater anti-inflammatory response through M2 macrophage polarization and driving T cells to a Th2 phenotype (Fan et al. 2012). The IFNγ expression construct was, similarly, shown to promote a more regenerative immune response and was also shown to reduce T cell activation in co-culture (Chen et al. 2015). Conversely, no changes to cell homing were reported in the IFNγ group.

Ex vivo lung perfusion (EVLP) is a technique designed to increase the available pool of donor lungs for transplantation by providing a platform to test the functionality of typically discarded lungs (Machuca and Cypel 2014). Conventional transplant requires specific criteria for donor lungs while much of the available pool, not meeting these criteria, are discarded without any functional assessment. Moreover, EVLP can, in fact, improve lung condition during the assessment period. One previously unavailable benefit to this technique is that it allows for modification of the lungs during ex vivo perfusion. Accordingly, treatment with UC-MSCs has been explored as a mechanism to reduce inflammation from ischemia-reperfusion injury as well as decrease the host alloimmune response following transplantation (Mordant et al. 2016). From early ex vivo experiments measuring the effects of human UC-MSC perfusion into pig lungs damaged by prolonged cold ischemic storage, Mordant et al. found modest functional improvements in the form of enhanced static compliance of these damaged lungs. Moreover, a significant

decrease of the pro-inflammatory cytokine, IL-8, within the lung perfusate was observed along with increased parenchymal VEGF. Although these are only early findings, they show a great deal of promise for enhancing the outcomes of EVLP reliant lung transplantations.

Sepsis can develop from a range of uncontrolled infections, which lead to specific antigens entering the systemic circulation, inducing an excessive and destructive immune response (Cohen 2002). If this is not controlled, it can result in organ failure. Furthermore, sepsis is the most common cause of acute respiratory distress syndrome (ARDS) which is associated with high patient mortality (Kim and Hong 2016). In both cases, systemic delivery of MSCs has been investigated to produce a long-term immunomodulatory environment within the host. Initially, BM-MSCs were compared to UC-MSCs in a cecal ligation and puncture model of sepsis (Chao et al. 2014). While treatment with UC-MSCs led to the greatest survival compared to BM-MSCs and PBS control, none of the differences were significant. Conversely, UC-MSCs, and to an equal extent, BM-MSCs, led to increased levels of circulating regulatory T cells, and higher regulatory T cell/T cell ratios. Additionally, circulating levels of IL-6 and TNFα were decreased following treatment with MSCs. In a second study using the same model, UC-MSCs were shown to only improve survival when co-administered with antibiotic, when compared to a PBS plus antibiotic treated control (Wu et al. 2016). Consistent with the previous study, TNFα and IL-6 levels were significantly reduced in the UC-MSC administered animals compared to PBS controls, along with MCP-1 and IFNγ. Moreover, the anti-inflammatory cytokine IL-10 was upregulated in the UC-MSC group. These data display the strong systemic anti-inflammatory capabilities of UC-MSCs in these models. UC-MSCs have also shown efficacy in treating animal models of bacterial associated ARDS (Masterson et al. 2015). In a model of LPS-induced ARDS, Sun et al. showed an increase in inflammatory cytokines IFNγ, TNFα, and MIP-2 following intratracheal administration of LPS (Sun et al. 2011). Each of these were significantly reduced in both plasma and bronchoalveolar lavage (BAL) fluid in groups treated with UC-MSCs 4 h after administration of LPS. Conversely, IL-10 was significantly increased in both BAL and plasma samples by delivery of MSCs. These changes are further linked to increased levels of circulating regulatory T cells, providing strong evidence that UC-MSCs are modifying the balance of pro- and anti-inflammatory agents in the immune response. Most importantly, however, these changes are met with significantly improved survival of UC-MSC treated mice. These results were further corroborated by Li et al., investigating intraperitoneally delivered LPS to induce ARDS. Similarly they found an increase in circulating levels of pro-inflammatory cytokines TNFα, IL-1β, and IL-6 by 6 h post LPS injection, which were all significantly reduced by administration of UC-MSCs 1 h after treatment with LPS (Li et al. 2012). Alternatively, whereas Sun et al. found an increase in IL-10 in both BAL fluid and plasma (Sun et al. 2011), Li et al. show that treatment with LPS increases IL-10 expression, which is then unchanged by the presence of MSCs. Treatment with UC-MSCs also resulted in a modest decrease in immune cell infiltrate and a slight improvement to histological scores. However, most notably, animals treated with UC-MSCs, again, displayed a significant

improvement in survival compared to the LPS treatment alone. Finally, in a recent study, Curley et al. (2017) investigated the idea of an off-the-shelf, xeno-free UC-MSC therapy for the treatment of ARDS (Curley et al. 2017). In this study, UC-MSCs were expanded under xeno-free conditions, frozen, thawed, and directly transferred into PBS for delivery into a rat, *E. coli* induced ARDS model. These data were also compared to BM-MSCs grown in standard xenogeneic conditions. Most notably, UC-MSCs reduced bacterial load, decreased neutrophil influx, and improved functional outcomes of lungs, compared to untreated controls, and were not different compared to BM-MSCs. Additionally, in a longitudinal study, treatment with UC-MSCs significantly improved survival of afflicted animals from 60% in the untreated group to 80% in the UC-MSC treated group. Treated animals also presented decreased TNFα and IL-6, and increased IL-10 within the BAL fluid, supporting the anti-inflammatory shift noted by others. However, most importantly, this study highlights the clinical potential for UC-MSCs in the treatment of ARDS. In addition to the numerous benefits associated with UC-MSCs in terms of isolation and expansion, Curley et al. provide support for their potential as an off-the-shelf, xeno-free treatment option, which is as effective in this model as BM-MSCs.

4.2 Wound Healing and Fibrosis

Both wound healing and development of organ fibrosis follow a cascade of processes beginning with inflammation and, unfortunately, generally ending with the formation of fibrotic scar tissue. This imperfect repair mechanism is rapid and robust, but depending on the severity of resulting fibrosis, can impact organ function. Once again, immunological effects have a critical role in the development of scar tissue, but stromal cell recruitment and activation, as well as angiogenesis are additional components involved in tissue repair.

There have been several investigations into the use of UC-MSCs, either alone or in combination with biomaterials, for treatment of wounds in animals, all showing significant positive effects of treatment. Critically, delivery of UC-MSCs into excisional wounds of immune compromised mice has been shown to significantly improve the rate of healing (Zebardast et al. 2010; Shohara et al. 2012; Fong et al. 2014). Moreover, this has been met with a more robust granulation tissue evidenced by enhanced tensile strength, increased granulation tissue thickness, increased collagen deposition, increased angiogenesis, and evidence of more mature vasculature (Zebardast et al. 2010; Shohara et al. 2012; Fong et al. 2014). Similar improvements were also noted in wounds in diabetic db/db mice (Fong et al. 2014), and surprisingly, when either immune compromised or diabetic animals were treated with conditioned media in place of UC-MSCs, significant beneficial effects were still observed (Shohara et al. 2012; Fong et al. 2014). Addition of UC-MSCs to complement other therapeutic strategies has also been investigated. Zhang et al. looked to combine UC-MSCs with skin fragment microparticles and pieces of Wharton's Jelly, showing qualitatively assessed regeneration compared to scar formation in the control wounds (Zhang et al. 2012b). Combination of UC-MSCs or conditioned

media with a polycaprolactone-(PCL) based scaffold incorporating aloe vera also showed evidence of regenerative effects over controls (Tam et al. 2014). The ability to add a biological component to existing biomaterials gives UC-MSCs a great potential for wound repair. The benefits of UC-MSCs are exemplified in a clinical setting where they have been applied, successfully, and without negative symptoms, to chronic wounds in dogs (Ribeiro et al. 2014). Two dogs displaying chronic wounds of duration 16 months and 24 months, both unresponsive to standard treatment, were treated with UC-MSCs delivered in poly(vinyl) alcohol (PVA) membranes. Two months following the initiation of treatment, the wounds on both dogs had re-epithelialized. The evidence provided by these studies emphasizes the pleiotropic effects of UC-MSCs on several responses involved in the repair mechanism including inflammation, granulation tissue formation, angiogenesis, and re-epithelialization.

Much like in the skin, injury to internal organs requires an acute response to ameliorate damage and return function to the organ, but again this response often leads to a detrimental fibrotic repair over time. Here we will discuss studies that have investigated the use of UC-MSCs in the acute and chronic responses of organ injury.

Acute kidney injury can result from various stimuli including chemical insults, ischemia, or physical obstruction. The homeostatic response to these stimuli is often excessive, leading to further tissue damage and impaired organ function. Several studies have looked to the potent paracrine effects of UC-MSCs to shift the healing response towards a more regenerative phenotype. An initial study by Cao et al. (2010) investigating ischemia reperfusion induced acute kidney injury in immunocompetent rats showed increased tubular epithelial cell proliferation and reduced apoptosis throughout the tissue (Cao et al. 2010). Moreover, these changes were concurrent with improved renal function as measured by reduced serum creatinine and blood urea nitrogen levels. These promising results have been supported in several further studies, many of which were aimed at identifying mechanisms for these improvements. While providing benefits to the acute phase of healing, Du et al. (2012) provide additional evidence showing reduced fibrosis in UC-MSC treated groups up to 22 weeks post ischemia reperfusion injury (Du et al. 2012). In a follow up study, this reduced fibrosis was linked to a shift in growth factor production, with an increased and prolonged expression of HGF at early time points and decreased TGFβ1 expression at later time points (Du et al. 2013). While these positive results were concurrent with reduced expression of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines, other models have been used to investigate acute kidney injury showing that the positive effects are independent of cytokine levels. In an immunocompromised rat model of folic acid-induced acute kidney injury, Fang et al. (2012) investigated changes in both human-derived and rat-derived pro- and anti-inflammatory cytokines, with no differences noted between UC-MSC treated and control groups (Fang et al. 2012). The authors attribute these discrepancies with previous work to their immunocompromised model, which still showed marked functional improvements when treated with UC-MSCs. Thus, it is possible that the changes in cytokine expression seen in immunocompetent animals may be an artifact of the host immune response and independent of tissue injury;

however, further investigation is required. In their study, Fang et al. (2012) attribute improvements to reduced apoptosis through the mitochondrial apoptotic pathway, mediated by caspase 9. While these studies provide strong support for functional improvements, they report only very low levels of cell engraftment into the tissue, suggesting that these cells are likely acting through endocrine processes. Whereas a cytokine-mediated response is controversial, investigation into UC-MSC secreted exosomes has suggested that these may mediate UC-MSC induced functional improvements (Zhou et al. 2013b). Concentrated UC-MSC derived exosomes injected locally into the kidney yielded improvements that were not seen in UC-MSC conditioned media nor in lung fibroblast derived exosome treated groups. While there is still a need to further elucidate the mechanisms of action, in a recent study Liu et al. (2016a) have expanded upon these findings by modifying UC-MSCs with retroviral delivered IGF-1. In a nude rat model of gentamicin-induced kidney injury modified UC-MSCs were shown to improve MSC homing to the injured kidney, to decrease inflammation and to reduce the amount of apoptosis over unmodified UC-MSCs. These data display hopeful results for UC-MSCs as a treatment of acute kidney injury, yet they also emphasize the critical shortcomings of rodent models, in which neither immune competent nor immune compromised animals provide a proxy for human disease. Nevertheless, these studies point in a promising direction.

Damage to the liver parenchyma via acute or chronic stimuli can lead to a potent immune response, resulting in excessive matrix production involving myofibroblastic differentiation of hepatic stellate cells (Xu et al. 2012). If the fibrotic response is not abrogated, cirrhosis can develop, characterized by architectural changes and vascular deficiency within the liver. Treatment of chemically induced liver fibrosis with UC-MSCs in animal models has shown promising results with both functional and structural improvements being noted. An initial study by Tsai et al. (2009) reports a vast decrease in collagen accumulation, and lesser myofibroblast differentiation as noted by a decreased αSMA content in UC-MSC treated groups (Tsai et al. 2009). These structural changes were also met with functional improvements assessed by specific enzyme levels in serum as biomarkers of hepatic health. In a time-course analysis, Lin et al. (2010) found that a reduced fibrotic score, evident following chemically induced liver fibrosis, could be detected at 14 and 21 days post UC-MSC injection, although was not evident by 7 days post injection (Lin et al. 2010). Conversely, control groups showed no improvement; however, it should be noted that no groups displayed functional improvements in the time frame investigated. These data have been further expanded by investigating dose response of UC-MSCs on treatment of liver fibrosis. Hong et al. (2014) show that an increased number of injections over time is superior for reducing collagen accumulation over larger doses delivered in a single injection (Hong et al. 2014). These initial findings have since been corroborated by additional studies investigating models of both chemically (Chai et al. 2016; Ma et al. 2016) and biologically (Hammam et al. 2016) induced liver fibrosis.

Acute lung injury of various etiologies can lead to the development of fibroblastic foci, leading to vast structural impairments to the pulmonary parenchyma.

While the development of ARDS (discussed above) can also lead to lung fibrosis, following a similar path from inflammation through to excessive matrix production, here we will focus on chemically induced acute lung injury. The first study to investigate UC-MSCs in a bleomycin-induced lung fibrosis model, by Moodley et al. 2009, showed that UC-MSCs were capable of homing specifically to fibrotic areas within the lung, whereas they were sparser in less damaged areas (Moodley et al. 2009). Moreover, UC-MSCs did not home to the lungs in control groups without bleomycin induced damage. Whole lung tissue, processed for mRNA transcript also yielded a lower expression of IFNγ, TGFβ1, MIF, TNFα, and IL-10, suggesting a broad spectrum of regulation on both pro-fibrotic and pro-inflammatory cytokines. These changes were concurrent with reduced collagen deposition, and histological improvements which lasted until the termination of the study. There has since been limited investigation of UC-MSCs in models of lung fibrosis, with one group investigating modified UC-MSCs to improve their anti-fibrotic efficacy. Using angiotensin-converting enzyme II (ACE II) transfected UC-MSCs, Min et al. (2014) show that these modified MSCs can, in fact, reduce collagen deposition further than unmodified UC-MSCs (Min et al. 2014). Additionally, inducing apoptosis in UC-MSCs through serum starvation was shown to have a positive effect on lung fibrosis, beyond the effect of normal UC-MSCs (Liu et al. 2016c). Interestingly, the apoptotic enriched population reduced neutrophil infiltrate and reduced damage even more so than UC-MSCs alone.

In a model of chronic pancreatitis induced by dibutyltin dichloride (DBTC), Zhou et al. (2013a) investigated the effect of intravenous delivery of UC-MSCs. Homing of UC-MSCs to the pancreas of injured animals was noted at 14 days and 28 days post injection, but was not noted in uninjured animals injected with cells (Zhou et al. 2013a). Importantly, an investigation into the biodistribution of UC-MSCs in several organs yielded an inability to detect UC-MSCs within the lungs or kidneys of injured, injected mice; however, there was accumulation within the liver, which importantly also showed signs of injury from the DBTC injection. Conversely, in the uninjured group, some UC-MSCs had accumulated in the lungs, but were not seen in the kidney or liver. Critically, reduced immune cell infiltrate and reduced fibrosis were also seen in animals treated with UC-MSCs, correlating with improved functional outcomes in these animals.

4.3 Bone and Joint Repair

Much like their role in repair of soft tissues, UC-MSCs have been shown to improve healing of bone defects. Todeschi et al. (2015), investigated both UC-MSCs and BM-MSCs in the repair of calvarial bone in mice, and while both improved repair to a similar extent, the mechanisms were distinct (Todeschi et al. 2015). Initially when implanted subcutaneously on a ceramic scaffold, BM-MSCs formed ectopic bone of human origin whereas UC-MSCs only induced a dense fibrous tissue, of mouse origin. This is in comparison to the scaffold alone which induced formation of a looser fibrous tissue. Still, when either cell was

applied to a calvarial defect, bone regeneration was significantly improved over the scaffold alone and was not different between BM-MSCs or UC-MSCs. Moreover, whereas human-derived cells could be identified to have differentiated into the bone in BM-MSC treated defects, this was not evident in the UC-MSC treated groups. Thus, while both treatment options led to improvements to bone regeneration, BM-MSCs directly contributed to bone formation whereas UC-MSCs acted only through paracrine effects. In two additional studies, directed differentiation studies were carried out in vitro prior to assessment of MSCs in vivo. Investigating induced pluripotent stem cells (iPSCs), BM-MSCs, and UC-MSCs in the repair of a damaged calvarial bone of rats, Wang et al. (2015) first showed differences in directed bone differentiation between different MSC origins (Wang et al. 2015). Importantly, while there were moderate differences in the extent of bone differentiation in vitro, with iPSCs tending to show decreased differentiation, all cell-treated groups performed equally in vivo. All cells, delivered on a calcium phosphate-based biomaterial, formed dense bone, and induced angiogenesis, to equal levels which were not observed in the biomaterial only control group. In fact, the only difference between cell-treated groups was that iPSCs stimulated slightly better integration with the native bone. Similarly, Kajiyama et al. (2015) found in their cell populations that UC-MSCs tended to underperform in bone-directed differentiation assays compared to BM-MSCs (Kajiyama et al. 2015). Conversely, in vivo only UC-MSCs significantly increased the bone volume of calvarial defects, whereas BM-MSCs had an intermediate effect, which was not different from the untreated control. These discrepancies between in vitro differentiation and in vivo performance reported by Wang et al. and Kajiyama et al. stress the importance of identifying meaningful potency assays for optimal cell selection for a given indication (Ujiie et al. 2015). Moreover, as paracrine effects, and not direct differentiation, are most often cited as the therapeutically relevant contribution of MSCs, analysis of differentiation as outlined in the ISCT's minimal criteria for defining MSCs (Dominici et al. 2006), likely does not relate to a population's ability to contribute to any malady in vivo.

UC-MSCs have also shown utility in the treatment of tendon injury. In a model of collagenase induced injury of the Achilles tendon in nude rats, Emrani and Davies (2011) show that injected UC-MSCs incorporated into the injured tendon and increase the mRNA expression of type I collagen within the tissue (Emrani 2011). Furthermore, mechanical testing of the tendon 30 days after injury revealed that UC-MSC treatment improved tensile strength from 41% of uninjured tendon in the control group up to 68% of uninjured tendon.

To our knowledge, human UC-MSCs have only been investigated in one study for the treatment of osteoarthritis (Saulnier et al. 2015). In a rabbit model of medial meniscal release induced osteoarthritis, UC-MSCs were injected at either 3 or 15 days following induction and rabbits were examined for gross morphological, histological, and gene expression changes. Only modest visual improvement was seen by 56 days post injury in groups treated with UC-MSCs on day 3 post injury. Surprisingly, inflammatory cell infiltrate was only seen in UC-MSC injected animals and persisted until day 56 which is possibly mediated by the upregulation of both

pro-inflammatory TNFα and anti-inflammatory IL-10 by UC-MSCs. Still, only injection of UC-MSCs at the earlier time point led to the upregulation of COL2a in the cartilage by 56 days, suggesting that cartilage repair and remodeling may be supported by injection of UC-MSCs.

4.4 Ischemia

Unilateral femoral artery ligation to promote hind limb ischemia in rodents is a widely used model to study critical limb ischemia. Grounded on the pro-angiogenic nature of MSCs, strategies to promote the growth of neovasculature to mitigate ischemia using these cells and their secreted products have been developed. Zhang et al. (2012a) first showed that UC-MSCs could be used to enhance angiogenesis and improve circulation within the ischemic hind limb, through a mechanism mediated by the release of microvesicles. In fact, concentrated microvesicles isolated from culture media could reestablish circulation within the ligated vasculature to the same extent as injected UC-MSCs. Others have expanded upon these initial findings by modifying UC-MSCs in culture to improve recovery of blood flow following ligation. Shen et al. (2013) investigated the use of endothelial progenitor cells, derived from UC-MSCs, to improve hind limb ischemia (Shen et al. 2013). These cells enhanced angiogenesis, and decreased both muscle degeneration and the extent of apoptosis in the ischemic tissue. Critically, these changes were sufficient to improve overall blood flow within the limb and improve functional recovery of the limb. Finally, Han et al. (2016) have shown that hypoxia preconditioned UC-MSCs injected into the hypoxic muscle tissue following femoral artery ligation, enhance the pro-angiogenic effect of UC-MSCs grown in normoxic conditions, increasing both the capillary density and the ratio of capillaries to muscle fibers (Han et al. 2016).

4.5 Myocardial Infarction

Temporary ligation of the left anterior descending (LAD) branch of the coronary artery is a common method to induce myocardial infarction in animal models, creating an ischemic area near the apex of the heart. Instead of a regenerative process to replace the damaged cardiomyocytes, a cascade of events follows such an ischemic episode that leads to the formation of scar tissue, impairing organ function. Initially, Dayan et al. (2011) investigated how MSC-induced changes in the immune response following myocardial infarction could modify outcomes in mice (Dayan et al. 2011). Both UC-MSCs and BM-MSCs, delivered intravenously, decreased total number of circulating macrophages following injury, with BM-MSCs also decreasing macrophage localization in the heart. Furthermore, the macrophages present showed greater polarization to the M2 anti-inflammatory phenotype, which the authors link to paracrine secretion of IL-10 evident in animals treated by either UC-MSC or BM-MSCs. MSC treatment also led to a decrease in the number of apoptotic cardiomyocytes, yet did not induce an

angiogenic response. Only a minor improvement to fractional shortening was evident at early timepoints in animals treated with MSCs, but this was not significantly different to control animals by 16 weeks post injury. Other functional parameters were not different between groups. Critically, although overall health of the animals treated with MSCs was slightly improved as evident by decreased lung congestion, no improvements to survival were seen (Dayan et al. 2011). Thus, while MSCs could modify the initial innate immune response, this alone was insufficient to improve outcomes in this model of myocardial infarction. To expand upon these data, Yannarelli et al. (2013) investigated an alternative MSC delivery strategy via intramyocardial injection (Yannarelli et al. 2013). Whereas intravenous delivery of UC-MSCs and BM-MSCs led primarily to localization within the lungs and did not affect cardiac function, local delivery of MSCs significantly improved fractional shortening, with UC-MSCs providing a superior response over BM-MSCs. Conversely, neither septum thickness nor other gross structural parameters were altered by MSC treatment. While neither MSC-treated groups displayed altered scar tissue formation, only treatment with UC-MSCs enhanced the angiogenic response over the control group (Yannarelli et al. 2013). Santos Nascimento et al. (2014) have corroborated these data, finding that treatment with UC-MSCs, delivered through intramyocardial injection, decreased the number of apoptotic cells, and modestly increased CD31+ cell infiltrate in the infarcted region, but no functional improvements, including fractional shortening, were resolved (Santos Nascimento et al. 2014). Further, in a miniswine model of myocardial infarction, Zhang et al. (2013) observed moderate functional and structural improvements following intramyocardial delivery of UC-MSCs (Zhang et al. 2013b). These were associated with greatly improved angiogenesis and a reduced number of apoptotic cells. Additionally, quantification of cell marker expression suggested that UC-MSCs induced infiltration and activation of cardiac stem cells in the ischemic tissue, potentially leading to a more regenerative phenotype. Consequently, these investigations into the utility of UC-MSCs for the treatment of acute myocardial infarction have yielded variable results. While UC-MSC treated groups display positive effects at the cellular level, these are not always met with functional improvements, suggesting that although they may ameliorate specific aspects of the natural response, they may be more beneficial when combined with other treatment modalities. Conversely, a recent study by Liu et al. (2016), investigating chronic myocardial ischemia in a porcine model, shows promise for UC-MSCs in this indication (Liu et al. 2016b). Four weeks prior to UC-MSC delivery, an ameroid constrictor was placed in the left coronary artery to restrict blood flow. In the follow up period, which lasted an additional four weeks after cell delivery, pigs receiving UC-MSCs displayed improvements in a range of functional parameters, including decreased heart rates, and improved ejection fraction and fractional shortening (Liu et al. 2016b). Moreover, treatment with UC-MSCs resulted in an improved angiogenic response, decreased apoptosis, and decreased fibrosis.

4.6 Diabetes

Although several secondary maladies can develop over time in diabetic patients, here we will focus solely on the aspects of insulin-mediated control of blood glucose.

Several studies have investigated the use of pancreatic β-like cells derived from UC-MSC progenitors to treat chemically induced type 1 diabetes (Kadam and Bhonde 2010; Tsai et al. 2012; Wang et al. 2014b). In all cases the differentiation protocols used resulted in a significantly, and greatly increased expression of both insulin and C-peptide, suggesting a β-like population. In these studies, UC-MSC-derived cells were delivered intravenously (Tsai et al. 2012), within a polymer capsule (Kadam and Bhonde 2010), or into the renal capsule (Wang et al. 2014b), and in all cases a reduced resting blood glucose was noted compared to untreated animals with induced diabetes. Although Kadam and Bhonde (2010) reported improvements equal to the level of non-diabetic animals, both Tsai et al. (2012) and Wang et al. (2014c) showed an intermediate response that remained worse than non-diabetic animals.

Type 2 diabetes has been approached in two studies investigating different aspects of the disease. Hu et al. (2014) first investigated a high fat diet, low dose streptozotocin induced type 2 mimicking regime in rats (Hu et al. 2014). In their model, treatment with UC-MSCs in combination with sitagliptin, an anti-diabetic drug targeting dipeptidyl peptidase 4, decreased fasting plasma glucose to the level of control, non-diabetic mice, an improvement over either treatment individually. Furthermore, they displayed recovery and regeneration of depleted pancreatic β-cells, a response also evident in UC-MSCs alone. Still, the mechanisms through which these two treatment modalities interact is uncertain. While regeneration of β-cells is a key component of type 2 diabetes treatment, insulin sensitization, especially in adipose tissue is equally important. Using the same model of type 2 diabetes, Xie et al. (2016) investigated the effect of UC-MSC delivery on the inflammatory state of endogenous adipose tissue to recover insulin sensitivity (Xie et al. 2016). Intravenous delivery of UC-MSCs significantly reduced resting blood glucose, in addition to improving glucose lowering following challenge via an intraperitoneal glucose tolerance test, although in both cases, not to the extent of non-diabetic animals. UC-MSC treated animals also displayed a decreased insulin resistance as measured by the product of the resting blood glucose and resting blood insulin levels compared to untreated diabetic animals. These changes were concomitant with greatly increased expression of anti-inflammatory M2 macrophage markers within the adipose tissue, and a decreased expression of pro-inflammatory M1 macrophage markers. Thus, it seems that treatment of type 2 diabetes with systemic dosing of UC-MSCs provides a two-pronged approach targeting both insulin resistance and insulin production through β-cell regeneration, indicating promise for future studies.

4.7 Cancer

Human cancer xenografts offer a unique tool to study relevant cancer cell growth kinetics under various pressures in a living model system. Using the MDA-231 breast cancer cell line, tumors rapidly develop in the lungs of SCID mice following delivery via tail vein injection (Ayuzawa et al. 2009). However, UC-MSCs injected weekly starting 8 days after MDA-231 cells, homed to tumors within the lungs and

significantly attenuated tumor growth following 3 weekly UC-MSC injections. In a second study, Wu et al. (2013) provide evidence that microvesicles released from UC-MSCs may be mediating this response (Wu et al. 2013). Using a T24 bladder cancer cell xenograft in nude mice, UC-MSCs or UC-MSC-derived microvesicles, injected alongside T24 cells, both significantly reduced tumor growth by 30 days, yet the concentrated microvesicles outperformed UC-MSCs. In both treatment groups, decreased cell proliferation and increased apoptosis within the tumors were noted. Still, the mechanisms through which the microvesicles are functioning in this system are unknown. It should also be noted that UC-MSCs have been shown to enhance tumor development, growth and metastasis in xenograft models. In an esophageal carcinoma cell line, Eca109, UC-MSCs increased tumor growth when injected simultaneously to cancer cells in a nude mouse xenograft model (Yang et al. 2014). Furthermore, when injected after tumors had already been established, enhanced tumor growth and metastasis to the lymph nodes were observed in UC-MSC treated groups. It is possible that there are type-specific interactions between cancer cells and UC-MSCs, and thus there is a need for investigation into these responses. Conversely, an alternative approach has recently been investigated for the use of UC-MSCs as therapeutics in cancer treatment. Using the natural homing nature of UC-MSCs Yan et al. (2016) have modified these cells to produce a soluble TRAIL ligand to induce apoptosis in established lung tumors of A549 lung cancer cell xenografts (Yan et al. 2016). Although the level of apoptosis within the tumor was greatly increased by the modified UC-MSCs, no difference in tumor size or animal survival were noted compared to the control groups. However, this approach permits the possibility to include additional modifications to improve upon these effects moving forward.

4.8 Antibody Therapy and Biodefense

The immune-privileged nature of UC-MSCs, allowing them to persist in immune competent allogeneic hosts for unprecedented duration, offers a unique system for delivery of biological payloads. Recently, Braid et al. (2016) published data in immune-compromised mice showing that the delivery and release of a prophylactic antibody could be delivered by transfected UC-MSCs, with the antibody remaining at a predicted protective level for up to 38 days following delivery and above the detectable limit for up to 109 days (Braid et al. 2016). In this study, an anti-Venezuelan equine encephalitis virus (VEEV) antibody delivered via transduced UC-MSCs into mice 10-days or 24 h prior to exposure to lethal doses of VEEV, greatly improved survival of animals, and reduced disease score in those animals exposed. These improvements were noted compared to untreated controls as well as controls treated with purified anti-VEEV antibody. These exciting data hold great promise for a prophylactic therapy that could have implications in biodefense or disease outbreaks. Additionally, this technology could be used to treat patients requiring antibody-based therapies to provide prolonged delivery of clinically relevant doses of monoclonal antibodies.

5 UC MSCs as Therapeutics for Human Disease

As of January 2016, there were 109 clinical trials registered on the ClinicalTrials.gov database involving umbilical cord tissue cells either alone or in conjunction with another cell type, but excluding those employing solely umbilical cord blood derived cells. These include Phase 1, 2, and 3 trials and therefore the current number should more accurately be recorded as 68. Of these 53 are in China, and the rest in Panama (6), Korea (2), Turkey (2), Chile (1), Italy (1), Indonesia (1), Taiwan (1), and the USA (1). Registration in this database is voluntary and does not include all clinical trials involving umbilical cord derived cells, although we restrict our analysis here to registered trials.

The trials have been targeted at 14 broad groups of medical indications, as illustrated in Fig. 5. The preponderance of trials has targeted autoimmune, cardiovascular, hematological, hepatic, neurodegenerative, and orthopedic conditions, each of which commands approximately 10% of the total number. The first trial [NCT00951210] targeted autism. This and four other trials were initiated in China, in 2009, for liver cirrhosis, liver failure, systemic sclerosis, and Type1 diabetes. Examples of trials in subsequent years illustrate the increasing breadth of conditions targeted. Thus, three new liver cirrhosis trials, and another T1 diabetes trial, were

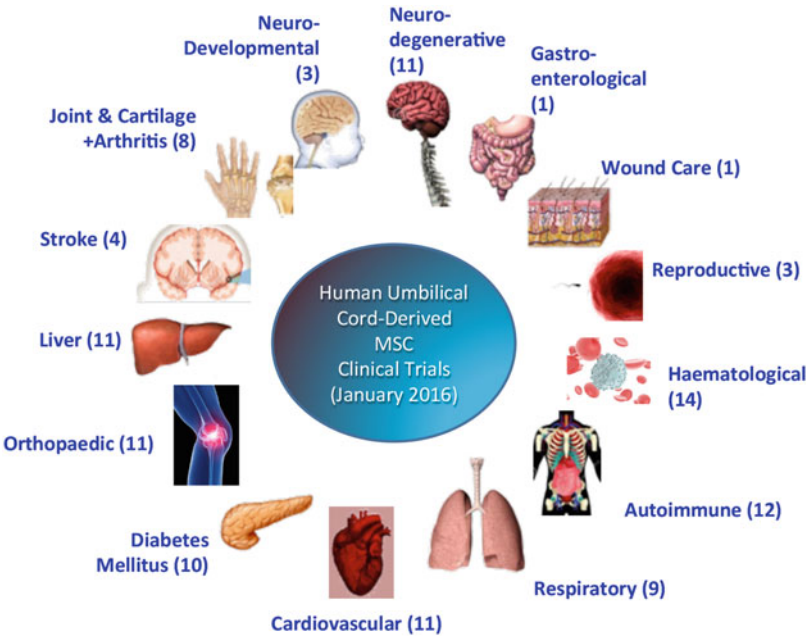


Fig. 5 The pictogram represents the range of clinical trials registered on ClinicalTrials.gov and employing human umbilical cord tissue derived MSC (excluding those from cord blood). This distribution is broken down in the pie-chart as percentages of the total per indication group. (Data courtesy of Tissue Regeneration Therapeutics Inc.)

added in 2010 among others that included broncopulmonary dysplasia and ulcerative colitis. 2011 saw enrollment in the first trials for stroke and Alzheimer's disease, in addition to acute myocardial infarction and T2 Diabetes. 2012 saw the first GvHD trial and co-administration with organ transplants, and two new trials targeting lupus. Both osteoarthritis and rheumatoid arthritis trials were added in 2013 together with MS and fracture non-union while 2015 saw three trials targeted at lung pathologies.

Since, the ClinicalTrials.gov data contains no details of the cell extraction methods employed it is not possible to compare the individual therapeutic products. However, some information is available in related publications. NCT01343511 reported an increased therapeutic effect of UCMSCs over cord blood mononuclear cells alone (Lv et al. 2013); NCT01360164 confirmed safety and a possible delay in the progression of spinocerebellar ataxia (Jin et al. 2013); NCT01741857 reported a satisfactory response in systemic lupus erythematosus (Wang et al. 2014a; Wang et al. 2014b); NCT01213186 reported improved host immune reconstitution in immune non-responders (Zhang et al. 2013a); and NCT01547091 showed that administration of umbilical cord cells with disease-modifying anti-rheumatic drugs provided persistent clinical benefits for patients with rheumatoid arthritis (Wang et al. 2013). It should be noted that none of these small studies was blinded, none were randomized and only some were controlled. Furthermore, while all studies used an intravenous route of administration, two also employed intrathecal delivery. The I.V. dose varied from 0.5×10^6 to 4×10^7cells/Kg body weight. Taken together, these studies do point to the administration of umbilical cord cells being safe and possibly providing some therapeutic benefit, although definitive information will not be available until large scale randomized, double blinded, placebo-controlled trials are undertaken.

6 Conclusions

If reaching clinical trials is the coming of age of a new cell therapeutic approach, then UC-MSCs can be considered to have reached this significant milestone. However, it remains to be seen – as with most attempts to employ MSCs from any tissue source – if robust and positive clinical outcomes can be achieved. Inevitably, progress in the employment of umbilical cord-derived mesenchymal cells is hampered by the lack of consensus on both anatomical descriptors of the cord tissue and standardization of cell isolation techniques. To promote progress, we consider it essential that both preclinical and clinical papers and reports provide sufficient methodological detail to allow repetition of the work by others in the field. This, after all, is considered the bedrock of good scientific reporting but, as we have shown herein, is lacking with respect to this increasingly important source of cells. Nevertheless, we remain optimistic that umbilical cord-derived mesenchymal stromal cells have great potential in providing therapeutic solutions for a large number of clinical indications.

References

Agah ME, Parivar K, Nabiuni M, Hashemi M, Soleimani M (2013) Induction of human umbilical Wharton's jelly-derived stem cells toward oligodendrocyte phenotype. J Mol Neurosci 51:328–336

Ayuzawa R, Doi C, Rachakatla RS, Pyle MM, Maurya DK, Troyer D, Tamura M (2009) Naïve human umbilical cord matrix derived stem cells significantly attenuate growth of human breast cancer cells in vitro and in vivo. Cancer Lett 280:31–37

Bosch J, Houben AP, Radke TF, Stapelkamp D, Bunemann E, Balan P, Buchheiser A, Liedtke S, Kögler G (2012) Distinct differentiation potential of "MSC" derived from cord blood and umbilical cord: are cord-derived cells true mesenchymal stromal cells? Stem Cells Dev 21:1977–88

Bouma G, Strober W (2003) The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol 3:521–533

Braid LR, Hu W-G, Davies JE, Nagata LP (2016) Engineered mesenchymal cells improve passive immune protection against lethal venezuelan equine encephalitis virus exposure. Stem Cels Transl Med 5:1–10

Can A, Karahuseyinoglu S (2007) Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. Stem Cells 25:2886–2895

Cao H, Qian H, Xu W, Zhu W, Zhang X, Chen Y, Wang M, Yan Y, Xie Y (2010) Mesenchymal stem cells derived from human umbilical cord ameliorate ischemia/reperfusion-induced acute renal failure in rats. Biotechnol Lett 32:725–732

Caplan AI (2007) Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 213:341–347

Chai N-L, Zhang X-B, Chen S-W, Fan K-X, Linghu E-Q (2016) Umbilical cord-derived mesenchymal stem cells alleviate liver fibrosis in rats. World J Gastroenterol 22:6036

Chao YH, Wu HP, Wu KH, Tsai YG, Peng CT, Lin KC, Chao WR, Lee MS, Fu YC (2014) An increase in CD3+CD4+CD25 + regulatory T cells after administration of umbilical cord-derived mesenchymal stem cells during sepsis. PLoS One 9:1–8

Chen Y, Song Y, Miao H, Xu Y, Lv M, Wang T, Hou Y (2015) Gene delivery with IFN-gamma-expression plasmids enhances the therapeutic effects of MSCs on DSS-induced mouse colitis. Inflamm Res 64:671–681

Cohen J (2002) The immunopathogenesis of sepsis. Nature 420:885–891

Coskun H, Can A (2015) The assessment of the in vivo to in vitro cellular transition of human umbilical cord multipotent stromal cells. Placenta 36:232–239

Curley GF, Jerkic M, Dixon S, Hogan G, Masterson C, O'Toole D, Devaney J, Laffey JG (2017) Cryopreserved, xeno-free human umbilical cord mesenchymal stromal cells reduce lung injury severity and bacterial burden in rodent. Escherichia coli–induced acute respiratory distress syndrome. Crit Care Med. 45:e202–e212

Davies JE, Walker JT, Keating AK (2017) Wharton's jelly: the rich, but enigmatic, source of MSCs. Stem Cells Transl Med 6(7):1620–1630

Dayan V, Yannarelli G, Billia F, Filomeno P, Wang XH, Davies JE, Keating A (2011) Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Basic Res Cardiol 106:1299–1310

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy 8:315–317

Donders R, Vanheusden M, Bogie JFJ, Ravanidis S, Thewissen K, Stinissen P, Gyselaers W, Hendriks JJA, Hellings N (2015) Human Wharton's jelly-derived stem cells display immunomodulatory properties and transiently improve rat experimental autoimmune encephalomyelitis. Cell Transplant 24:2077–2098

Du T, Cheng J, Zhong L, Zhao X, Zhu J, Zhu Y-J, Liu G-H (2012) The alleviation of acute and chronic kidney injury by human Wharton's jelly-derived mesenchymal stromal cells triggered by ischemia-reperfusion injury via an endocrine mechanism. Cytotherapy 14:1215–1227

Du T, Zou X, Cheng J, Wu S, Zhong L, Ju G, Zhu J, Liu G (2013) Human Wharton ' s jelly-derived mesenchymal stromal cells reduce renal fibrosis through induction of native and foreign hepatocyte growth factor synthesis in injured tubular epithelial cells. Stem Cell Res Ther 4:1

Emrani H, Davies JE (2011) Umbilical cord perivascular cells: A mesenchymal cell source for treatment of tendon injuries. Open Tiss Eng Regen Med J 4:112–119

Ennis J, Götherström C, Le Blanc K, Davies JE (2008) In vitro immunologic properties of human umbilical cord perivascular cells. Cytotherapy 10:174–181

Fan H, Zhao G, Liu L, Liu F, Gong W, Liu X, Yang L, Wang J, Hou Y (2012) Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-induced colitis. Cell Mol Immunol 9:473–481

Fang TC, Pang CY, Chiu SC, Ding DC, Tsai RK (2012) Renoprotective effect of human umbilical cord-derived mesenchymal stem cells in immunodeficient mice suffering from acute kidney injury. PLoS One 7:1–15

Farias VA, Linares-Fernández JL, Peñalver JL, Payá Colmenero JA, Ferrón GO, Duran EL, Fernández RM, Olivares EG, O'Valle F, Puertas A, Oliver FJ, Ruiz De Almodóvar JM (2011) Human umbilical cord stromal stem cell express CD10 and exert contractile properties. Placenta 32:86–95

Fong CY, Subramanian A, Biswas A, Gauthaman K, Srikanth P, Hande MP, Bongso A (2010) Derivation efficiency, vcell proliferation, freeze-thaw survival, stem-cell properties and differentiation of human Wharton's jelly stem cells. Reprod Biomed Online 21:391–401

Fong CY, Tam K, Cheyyatraivendran S, Gan SU, Gauthaman K, Armugam A, Jeyaseelan K, Choolani M, Biswas A, Bongso A (2014) Human Wharton's Jelly stem cells and its conditioned medium enhance healing of excisional and diabetic wounds. J Cell Biochem 115:290–302

Hammam OA, Elkhafif N, Attia YM, Mansour MT, Elmazar MM, Abdelsalam RM, Kenawy SA, El-Khatib AS (2016) Wharton's jelly-derived mesenchymal stem cells combined with praziquantel as a potential therapy for Schistosoma mansoni-induced liver fibrosis. Sci Rep 6:21005

Han KH, Kim AK, Kim MH, Kim DH, Go HN, Kim DI (2016) Enhancement of angiogenic effects by hypoxia-preconditioned human umbilical cord-derived mesenchymal stem cells in a mouse model of hindlimb ischemia. Cell Biol Int 40:27–35

Hong J, Jin H, Han J, Hu H, Liu J, Li L, Huang Y, Wang D, Wu M, Qiu L, Qian Q (2014) Infusion of human umbilical cord-derived mesenchymal stem cells effectively relieves liver cirrhosis in DEN-induced rats. Mol Med Rep 9:1103–1111

Hu J, Wang F, Sun R, Wang Z, Yu X, Wang L, Gao H, Zhao W, Yan S, Wang Y (2014) Effect of combined therapy of human Wharton's jelly-derived mesenchymal stem cells from umbilical cord with sitagliptin in type 2 diabetic rats. Endocrine 45:279–287

Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U (2007) Stem Cells Derived from Human Fetal Membranes Display Multilineage Differentiation Potential. Biol Reprod 77(3):577–588

Jin J, Liu Z, Lu Z, Guan D, Wang C, Chen Z, Zhang W, Wu J, Xu Y (2013) Safety and efficacy of umbilical cord mesenchymal stem cell therapy in hereditary spinocerebellar ataxia. Curr Neurovasc Res 10:11–20

Kadam SS, Bhonde RR (2010) Islet neogenesis from the constitutively nestin expressing human umbilical cord matrix derived mesenchymal stem cells. Islets 2:112–120

Kajiyama S, Ujiie Y, Nishikawa S, Inoue K, Shirakawa S, Hanada N, Liddell R, Davies JE, Gomi K (2015) Bone formation by human umbilical cord perivascular cells. J Biomed Mater Res Part A 103:2807–2814

Kim WY, Hong SB (2016) Sepsis and acute respiratory distress syndrome: recent update. Tuberc Respir Dis 79:53–57

Kita K, Gauglitz GG, Phan TT, Herndon DN, Jeschke MG (2010) Isolation and characterization of mesenchymal stem cells from the sub-amniotic human umbilical cord lining membrane. Stem Cells Dev 19:491–502

Li J, Li D, Liu X, Tang S, Wei F (2012) Human umbilical cord mesenchymal stem cells reduce systemic inflammation and attenuate LPS-induced acute lung injury in rats. J Inflamm 9:33

Liang L, Dong C, Chen X, Fang Z, Xu J, Liu M, Zhang X, Gu DS, Wang D, Du W, Zhu D, Han Z (2011) Human umbilical cord mesenchymal stem cells ameliorate mice TNBS-induced colitis. Cell Transplant 20:1395–1408

Lin S-Z, Chang Y-J, Liu J-W, Chang L-F, Sun L-Y, Li Y-S, Luo G-H, Liao C-H, Chen P-H, Chen T-M, Lee R-P, Yang K-L, Harn H-J, Chiou T-W (2010) Transplantation of human Wharton's Jelly-derived stem cells alleviates chemically induced liver fibrosis in rats. Cell Transplant 19:1451–1463

Lin Y, Lin L, Wang Q, Jin Y, Zhang Y, Cao Y, Zheng C (2015) Transplantation of human umbilical mesenchymal stem cells attenuates dextran sulfate sodium-induced colitis in mice. Clin Exp Pharmacol Physiol 42:76–86

Liu R, Zhang Z, Lu Z, Borlongan C, Pan J, Chen J, Qian L, Liu Z, Zhu L, Zhang J, Xu Y (2013) Human umbilical cord stem cells ameliorate experimental autoimmune encephalomyelitis by regulating immunoinflammation and remyelination. Stem Cells Dev 22:1053–1062

Liu P, Feng Y, Dong D, Liu X, Chen Y, Wang Y, Zhou Y (2016a) Enhanced renoprotective effect of IGF-1 modified human umbilical cord-derived mesenchymal stem cells on gentamicin-induced acute kidney injury. Sci Rep 6:20287

Liu C-B, Huang H, Sun P, Ma S-Z, Liu A-H, Xue J, Fu J-H, Liang Y-Q, Liu B, Wu D-Y, Lu S-H, Zhang X-Z (2016b) Human umbilical cord-derived mesenchymal stromal cells improve left ventricular function, perfusion, and remodeling in a porcine model of chronic myocardial ischemia. Stem Cells Transl Med 5:1004–1013

Liu F-B, Lin Q, Liu Z-W, Liu Z (2016c) A study on the role of apoptotic human umbilical cord mesenchymal stem cells in bleomycin-induced acute lung injury in rat models. Eur Rev Med Pharmacol Sci 20:969–982

Lu L, Zhao Q, Wang X, Xu Z, Lu Y, Chen Z, Liu Y (2006) Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica 91:1017–1026

Lv Y, Zhang Y, Liu M, Qiuwaxi J, Ashwood P, Cho SC, Huan Y, Ge R, Chen X, Wang Z, Kim B, Hu X (2013) Transplantation of human cord blood mononuclear cells and umbilical cord-derived mesenchymal stem cells in autism. J Transl Med 11:196

Ma J, Li L, Chen L, Wang X (2016) Effects of human umbilical cord mesenchymal stem cells in the treatment of CCl4-induced liver cirrhosis. Int J Clin Exp Med 9:5168–5180

Machuca TN, Cypel M (2014) Ex vivo lung perfusion. J Thoracic Disease 6:1054–1062

Maruyama Y (1963) The human endothelial cell in tissue culture. Zeitschrift fiir Zellforschung 60:69–79

Masterson C, Jerkic M, Curley GF, Laffey JG (2015) Mesenchymal stromal cell therapies: potential and pitfalls for ARDS. Minerva Anestesiol 81:179–194

McQualter JL, Bernard CCA (2007) Multiple sclerosis: a battle between destruction and repair. J Neurochem 100:295–306

Min F, Gao F, Li Q, Liu Z (2014) Therapeutic effect of human umbilical cord mesenchymal stem cells modified by angiotensin-converting enzyme 2 gene on bleomycin-induced lung fibrosis injury. Mol Med Rep 11:2387–2396

Moodley Y, Atienza D, Manuelpillai U, Samuel CS, Tchongue J, Ilancheran S, Boyd R, Trounson A (2009) Human umbilical cord mesenchymal stem cells reduce fibrosis of bleomycin-induced lung injury. Am J Pathol 175:303–313

Mordant P, Nakajima D, Kalaf R, Iskender I, Maahs L, Behrens P, Coutinho R, Iyer RK, Davies JE, Cypel M, Liu M, Waddell TK, Keshavjee S (2016) Mesenchymal stem cell treatment is associated with decreased perfusate concentration of IL-8 during ex vivo perfusion of donor lungs after 18 h preservation. J Heart Lung Transplant 35:1245–1254

Nanaev AK, Kohnen G, Milovanov AP, Domogatsky SP, Kaufmann P (1997) Stromal differentiation and architecture of the human umbilical cord. Placenta 18(1):53–64

Payne NL, Sun G, Mcdonald C, Layton D, Moussa L, Emerson-Webber A, Veron N, Siatskas C, Herszfeld D, Price J, Bernard CCA (2013) Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. Cell Transplant 22:1409–1425

Ribeiro J, Pereira T, Amorim I, Caseiro AR, Lopes MA, Lima J, Gartner A, Santos JD, Bártolo PJ, Rodrigues JM, Mauricio AC, Luís AL (2014) Cell therapy with human MSCs isolated from the umbilical cord Wharton Jelly associated to a PVA membrane in the treatment of chronic skin wounds. Int J Med Sci 11:979–987

Santos Nascimento D, Mosqueira D, Sousa LM, Teixeira M, Filipe M, Resende TP, Araújo AF, Valente M, Almeida J, Martins JP, Santos JM, Bárcia RN, Cruz P, Cruz H, Pinto-do-Ó P (2014) Human umbilical cord tissue-derived mesenchymal stromal cells attenuate remodeling after myocardial infarction by proangiogenic, antiapoptotic, and endogenous cell-activation mechanisms. Stem Cell Res Ther 5:5

Sarugaser R, Lickorish D, Baksh D, Hosseini M, Davies JE (2005) Human Umbilical Cord Perivascular (HUCPV) Cells: A Source of Mesenchymal Progenitors. Stem Cells 23(2): 220–229

Sarugaser R, Hanoun L, Keating A, Stanford WL, Davies JE (2009) Human mesenchymal stem cells self-renew and differentiate according to a deterministic hierarchy. PLoS One 4:e6498

Saulnier N, Viguier E, Perrier-groult E, Chenu C, Pillet E, Roger T, Maddens S, Boulocher C (2015) Intra-articular administration of xenogeneic neonatal Mesenchymal Stromal Cells early after meniscal injury down-regulates metalloproteinase gene expression in synovium and prevents cartilage degradation in a rabbit model of osteoarthritis. Osteoarthr Cartil 23:122–133

Schugar RC, Chirieleison SM, Wescoe KE, Schmidt BT, Askew Y, Nance JJ, Evron JM, Peault B, Deasy BM (2009) High harvest yield, high expansion, and phenotype stability of CD146 mesenchymal stromal cells from whole primitive human umbilical cord tissue. J Biomed Biotechnol 2009:789526

Shen W, Liang C, Wu V-C, Wang S-H, Young G, Lai I, Chien C, Wang S, Wu K, Chen Y (2013) Endothelial progenitor cells derived from Wharton's Jelly of the umbilical cord reduces ischemia-induced hind limb injury in diabetic mice by inducing HIF-1α/IL-8 expression. Stem Cells Dev 22:1408–1418

Shohara R, Yamamoto A, Takikawa S, Iwase A, Hibi H, Kikkawa F, Ueda M (2012) Mesenchymal stromal cells of human umbilical cord Wharton's Jelly accelerate wound healing by paracrine mechanisms. Cytotherapy 14:1171–1181

Steinman L (2014) Immunology of relapse and remission in multiple sclerosis. Annu Rev Immunol 32:257–281

Subramanian A, Fong C-Y, Biswas A, Bongso A (2015) Comparative characterization of cells from the various compartments of the human umbilical cord shows that the Wharton's Jelly compartment provides the best source of clinically utilizable mesenchymal stem cells. PLoS One 10: e0127992

Sun J, Han Z, Liao W, Yang SG, Yang Z, Yu J, Meng L, Wu R, Han ZC (2011) Intrapulmonary delivery of human umbilical cord mesenchymal stem cells attenuates acute lung (FOXP3) + regulatory T cells and balancing anti- and pro-inflammatory factors. Cell Physiol Biochem 27:587–596

Tam K, Cheyyatraviendran S, Venugopal J, Biswas A, Choolani M, Ramakrishna S, Bongso A, Fong CY (2014) A nanoscaffold impregnated with human Wharton's Jelly stem cells or its secretions improves healing of wounds. J Cell Biochem 115:794–803

Todeschi MR, El Backly R, Capelli C, Daga A, Patrone E, Introna M, Cancedda R, El Backly R, Capelli C, Daga A, Patrone E, Introna M, Cancedda R, Mastrogiacomo M (2015) Transplanted umbilical cord mesenchymal stem cells modify the in vivo microenvironment enhancing angiogenesis and leading to bone regeneration. Stem Cells Dev 24:1570–1581

Troyer DL, Weiss ML (2008) Concise review: Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 26:591–599

Tsai P, Fu T-W, Arthur Chen Y-M, Ko T, Chen T, Shih Y, Hung S, Fu Y (2009) The therapeutic potential of human umbilical mesenchymal stem cells from Wharton's Jelly in the treatment of rat liver fibrosis. Liver Transpl 15:484–495

Tsai P-J, Wang H-S, Shyr Y-M, Weng Z-C, Tai L-C, Shyu J-F, Chen T-H (2012) Transplantation of insulin-producing cells from umbilical cord mesenchymal stem cells for the treatment of streptozotocin-induced diabetic rats. J Biomed Sci 19:47

Ujiie Y, Gomi K, Davies JE (2015) MSC functional phenotype: Assay, age and source dependence. Sci Proc 2:1–5

Wang LL, Wang LL, Cong X, Liu G, Zhou J, Bai B, Li Y, Bai W, Li M, Ji H, Zhu D, Wu M, Liu Y (2013) Human umbilical cord mesenchymal stem cell therapy for patients with active rheumatoid arthritis: safety and efficacy. Stem Cells Dev 22:3192–3202

Wang D, Feng X, Lu LL, Konkel JE, Zhang H, Chen Z, Li X, Gao X, Lu LL, Shi S, Chen W, Sun L (2014a) A CD8 T cell/indoleamine 2,3-dioxygenase axis is required for mesenchymal stem cell suppression of human systemic lupus erythematosus. Arthritis Rheumatol 66:2234–2245

Wang G, Li Y, Wang Y, Dong Y, Wang FS, Ding Y, Kang Y, Xu X (2014b) Roles of the co-culture of human umbilical cord Wharton's jelly-derived mesenchymal stem cells with rat pancreatic cells in the treatment of rats with diabetes mellitus. Exp Ther Med 8:1389–1396

Wang D, Li J, Zhang Y, Zhang M, Chen J, Li X, Hu X, Jiang S, Shi S, Sun L, Wang D, Li J, Zhang Y, Zhang M, Chen J, Li X, Hu X, Jiang S, Shi S, Sun L (2014c) Umbilical cord mesenchymal stem cell transplantation in active and refractory systemic lupus erythematosus: a multicenter clinical study. Arthritis Res Ther 16:R79

Wang P, Liu X, Zhao L, Weir MD, Sun J, Chen W, Man Y, Xu HHK (2015) Bone tissue engineering via human induced pluripotent, umbilical cord and bone marrow mesenchymal stem cells in rat cranium. Acta Biomater 18:236–248

Wharton TW (1656) Adenographia. Translated by S. Freer 1996. Oxford University Press, Oxford, UK, p 243

Wu S, Ju GQ, Du T, Zhu YJ, Liu GH (2013) Microvesicles derived from human umbilical cord Wharton's Jelly mesenchymal stem cells attenuate bladder tumor cell growth in vitro and in vivo. PLoS One 8:1–12

Wu K-H, Wu H-P, Chao W-R, Lo W-Y, Tseng P-C, Lee C-J, Peng C-T, Lee M-S, Chao Y-H (2016) Time-series expression of toll-like receptor 4 signaling in septic mice treated with mesenchymal stem cells. Shock 45:634–640

Xie Z, Hao H, Tong C, Cheng Y, Liu J, Pang Y, Si Y, Guo Y, Zang L, Mu Y, Han W (2016) Human umbilical cord-derived mesenchymal stem cells elicit macrophages into an anti-inflammatory phenotype to alleviate insulin resistance in type 2 diabetic rats. Stem Cells 34:627–639

Xu R, Zhang Z, Wang FS (2012) Liver fibrosis: mechanisms of immune-mediated liver injury. Cell Mol Immunol 9:296–301

Yan C, Song X, Yu W, Wei F, Li H, Lv M, Zhang X, Ren X (2016) Human umbilical cord mesenchymal stem cells delivering sTRAIL home to lung cancer mediated by MCP-1/CCR2 axis and exhibit antitumor effects. Tumor Biol 37:8425–8435

Yang X, Li Z, Ma Y, Gao J, Liu S, Gao Y, Wang G (2014) Human umbilical cord mesenchymal stem cells promote carcinoma growth and lymph node metastasis when co-injected with esophageal carcinoma cells in nude mice. Cancer Cell Int 14:93

Yannarelli G, Dayan V, Pacienza N, Lee CJ, Medin J, Keating A (2013) Human umbilical cord perivascular cells exhibit enhanced cardiomyocyte reprogramming and cardiac function after experimental acute myocardial infarction. Cell Transplant 22:1651–1666

Zebardast N, Lickorish D, Davies JE (2010) Human umbilical cord perivascular cells (HUCPVC) A mesenchymal cell source for dermal wound healing. Organogenesis 6:197–203

Zhang H-C, Liu X-B, Huang S, Bi X-Y, Wang H-X, Xie L-X, Wang Y-Q, Cao X-F, Lv J, Xiao F-J, Yang Y, Guo Z-K (2012a) Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both in vitro and in vivo. Stem Cells Dev 21:3289–3297

Zhang Q, O'Hearn S, Kavalukas SL, Barbul A (2012b) Role of high mobility group Box 1 (HMGB1) in wound healing. J Surg Res 176:343–347

Zhang Z, Fu J, Xu X, Wang S, Xu R, Zhao M, Nie W, Wang X, Zhang J, Li T, Su L, Wang F-S (2013a) Safety and immunological responses to human mesenchymal stem cell therapy in difficult-to-treat HIV-1-infected patients. AIDS 27:1283–1293

Zhang W, Liu X, Yang L, Zhu D, Zhang Y, Chen Y, Zhang H-Y (2013b) Wharton's jelly-derived mesenchymal stem cells promote myocardial regeneration and cardiac repair after miniswine acute myocardial infarction. Coron Artery Dis 24:549–558

Zhou C, Li M, Qin A, Lv S-X, Wen-Tang, Zhu X-Y, Li L-Y, Dong Y, Hu C-Y, Hu D-M, Wang S-F (2013a) Reduction of fibrosis in dibutyltin dichloride-induced chronic pancreatitis using rat umbilical mesenchymal stem cells from Wharton's jelly. Pancreas 42:1291–1302

Zhou Y, Xu H, Xu W, Wang B, Wu H, Tao Y, Zhang B, Wang M, Mao F, Yan Y, Gao S, Gu H, Zhu W, Qian H (2013b) Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis in vivo and in vitro. Stem Cell Res Ther 4:34

Hair Follicle Stem Cells and Hair Regeneration

Sung-Jan Lin, Wen-Yen Huang, Chih-Chiang Chen, Mingxing Lei, and Jin-Bon Hong

Contents

S.-J. Lin (✉)
Department of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei, Taiwan

Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan

Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan

Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan
e-mail: drsjlin@ntu.edu.tw

W.-Y. Huang
Department of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei, Taiwan
e-mail: adamhuangcats@gmail.com

C.-C. Chen
Department of Dermatology, Taipei Veterans General Hospital, Taipei, Taiwan

Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan

Department of Dermatology, National Yang-Ming University, Taipei, Taiwan
e-mail: docs1.tw@yahoo.com.tw

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_12

Abstract

The hair follicle (HF), forming embryonically through cutaneous epithelial-mesenchymal interaction, is a sophisticated multifunctional organ. In addition to its protective, thermoregulatory, mechanosensory, and piloerectile functions, it is also essential for social communication. HFs repeatedly cycle through resting (telogen), growing (anagen), and regressing (catagen) phases in life. Prolonged arrest in telogen or disruption of anagen can result in unwanted hair loss or alopecia. Hair regeneration from telogen is fueled by hair follicle stem cells (HFSCs) located in the secondary hair germ and the bulge epithelium. HFSCs are subject to non-cell-autonomous regulation by their niche. HFSC niche cells can be categorized into modules of signaling, sensing, and message-relaying functions that enable HFSCs to adapt their regenerative activities according to varying physiological needs and environmental changes. Niche dysfunction can lead to alopecia. For example, androgenetic alopecia, i.e., male pattern hair loss, is caused by dysfunctional HF dermal papilla cells, while alopecia areata, i.e., spot baldness, results from infiltration of auto-reactive T cells into HFs. In addition to telogen-to-anagen regeneration, anagen HFs can adopt another regenerative scheme of anagen HF repair (AHFR) when damaged. Basal HF epithelial cells outside the HFSC compartments reserve a concealed progenitor property with plastic cell fate choices. By mobilizing these nonconventional basal progenitor cells, AHFR restores lost structures to resume the disrupted anagen and avoids premature catagen entry, as seen in the dystrophic anagen pathway following chemotherapeutic and radiotherapeutic injuries. Targeting disease-specific niche pathology and enhancing the intrinsic

M. Lei
111 Project Laboratory of Biomechanics and Tissue Repair, College of Bioengineering, Chongqing University, Chongqing, China
e-mail: mingxing@cqu.edu.cn

J.-B. Hong
Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan
e-mail: jbtouching@gmail.com

regenerative programs of AHFR can therefore help to develop new strategies for the treatment of hair loss.

Abbreviations

AHFR	Anagen hair follicle repair
HF	Hair follicle
HFSC	Hair follicle stem cell
IRS	Inner root sheath
ORS	Outer root sheath
SC	Stem cell

1 Introduction

The hair follicle (HF), often considered as a cutaneous appendage, is a tiny but extremely intricate organ with versatile functions in mammals (Schneider et al. 2009). The mammalian hair coat forms the outermost barrier to insulate the body from physical and chemical insults from the environment as well as to keep the body from temperature loss for thermal regulation. Incorporated with dense innervation, HFs are capacitated with mechanosensory functions (Cheng et al. 2018; Botchkarev et al. 1997). Ordered developmental integration of sympathetic nerves and arrector pili muscle further endows HFs with the unique piloerectile function (Botchkarev et al. 1997; Fan et al. 2018a; Fujiwara et al. 2011; Furlan et al. 2016), transforming HFs into a motor organ. In human, HFs, especially scalp hair and beards, have important ornamental functions that are essential for social communication. Unwanted hair loss can compromise senses of well-being and pose psychosocial distress to affected individuals (McGarvey et al. 2001). Treatments that can prevent hair loss from injuries and facilitate hair regeneration are therefore of high clinical significance.

As an integral part of skin (Chase 1954), hair growth and HF stem cells (**HFSC**s) are regulated not only by signals intrinsic to the HF but also by neighboring cells, systemic factors, and the external environment (Fan et al. 2018a; Chen et al. 2016, 2020; Hsu et al. 2014a). Pathological dysfunction of these regulatory networks can detrimentally inhibit hair growth. In addition to the reactivation of HFSCs to launch a new round of hair growth, promoting intrinsic reparative mechanisms of HFs following insults, such as inflammation and chemo-/radiotherapeutic injury, can maintain HF in the growing state to reduce hair loss (Huang et al. 2017a, 2019). When the entire HFs are lost, transplantation of remaining HFs to the region of loss is the main treatment choice (Orentreich 1959). Because this approach involves redistribution of remaining HFs without the creation of new HFs, it is not applicable to the treatment of severe hair loss. Promoting HF neogenesis by re-eliciting the developmental program of HFs is also a field of high interest in hair research (Fan et al. 2018b; Young et al. 2008;

Huang et al. 2013; Jahoda et al. 1984; Reynolds and Jahoda 1992; Abaci et al. 2018; Ito et al. 2007).

2 Hair Follicle Structure and Hair Cycle

A HF is composed of two main parts: epithelium of keratinocytes and mesenchyme of dermal papilla and dermal sheath cells (Figs. 1, 2) (Chase 1954; Philpott and Paus 1998; Rahmani et al. 2014). Compared with other organs, the HF is unique in its remarkable ability to undergo lifelong cyclic growth immediately after completion of its development. The hair cycle perpetuates through repeated bouts of telogen (resting phase), anagen (regeneration phase), and catagen (regression phase) (Fig. 2) (Paus and Cotsarelis 1999; Muller-Rover et al. 2001; Dry 1926). These three phases of the hair cycle run periodically during the lifetime.

In normal human scalp, most HFs are in the anagen phase any time in life (~90%) (Paus and Cotsarelis 1999). The anagen of scalp hair can persist for up to more than 30 years (Longest head hair (female) 2004), which is 600-fold longer than the duration of the anagen of mouse pelage HFs (~17 days) (Chase 1954; Chase et al. 1951). A mature anagen HF is a cylindrical structure which can be divided into the upper "permanent" segment and the lower "transient" or "transformable" segment (Muller-Rover et al. 2001; Murray et al. 2012; Ackerman et al. 1993). During hair cycle transition, the structure of the upper segment is constantly maintained, while the lower segment is periodically remodeled. The upper segment of an anagen HF consists of the infundibulum and the isthmus (Fig. 1a–c) (Ackerman et al. 1993). The quiescent or slow-cycling HFSCs are harbored in the lower isthmus, so-called bulge region, which is also the lower end of the permanent segment of an anagen HF (Fig. 1c) (Cotsarelis et al. 1990; Ohyama et al. 2006). At the base of the lower segment, the hair bulb containing matrix cells is connected with the bulge through a long stretch of suprabulbar HF epithelium of the stem (Fig. 1a, b, c) (Ackerman et al. 1993). The infundibulum, isthmus, bulge, stem, and hair bulb all belong to the parts of the anagen HF epithelium (Schneider et al. 2009; Ackerman et al. 1993).

In the cross section, the cylindrical architecture of an anagen HF comprises eight distinct concentric layers of cells with diverse differentiation (Fig. 1e, f), each of them expressing a distinct pattern of keratins (Porter et al. 2004). The outer layer lying on the basement membrane is the outer root sheath (**ORS**), and the inner layers include the companion layer, inner root sheath (**IRS**), and hair shaft (**HS**). Both IRS and HS contain three distinct sublayers: Henle's layer, Huxley's layer, and cuticle in IRS and hair cuticle, cortex, and medulla in HS (Schneider et al. 2009; Paus and Cotsarelis 1999; Ackerman et al. 1993). The companion layer located between ORS and IRS helps to connect them and might act as a shear plane to benefit the upward sliding of IRS along the immotile ORS (Fig. 1d, e) (Chase 1954). These internal structures are derived from the basal germinative cells in the hair matrix (Fig. 1e).

The dermal papilla (Fig. 1e), constituted in embryonic skin development, is a cluster of specialized fibroblasts located at the bottom of HFs (Philpott and Paus 1998; Sennett and Rendl 2012; Millar 2002). Dermal sheath cells envelop the outer

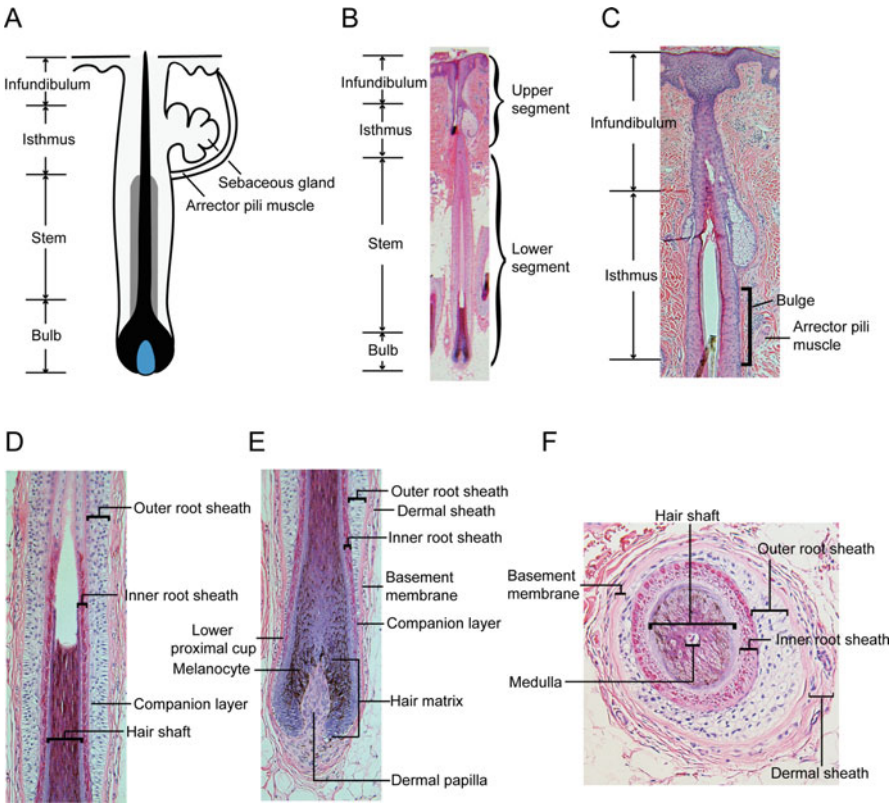


Fig. 1 Human HF structure. (**a**) Depiction of the human anagen HF structure. The anagen HF can be divided into an upper permanent segment and a lower transient segment. The upper permanent segment includes the infundibulum and the isthmus and the lower transient segment comprises a long stem and a hair bulb. Sebaceous glands are connected with the upper segment through the sebaceous duct. Arrector pili muscles attach to the lower end of the isthmus. (**b**) Vertical histological section of a human anagen HF. (**c**) The upper segment of a human anagen HF. The bulge epithelium is located at the lower part of the isthmus where the arrector pili muscles attach. (**d**) The stem. The hair shaft continues to mature and moves upward with the inner root sheath. (**e**) The hair bulb. The anagen dermal papilla exhibits a pear shape in the vertical section. The dermal papilla is enveloped by highly proliferative germinative cells of the matrix which give rise to all the internal structures from the hair shaft to the companion layer of the anagen HF. The lower proximal cup cells are basal cells continuous with the outer root sheath. They line the outer surface of the hair bulb, touching the basement membrane. (**f**) Horizontal section of the HF right above the hair bulb. The HF is surrounded by a dermal sheath across the basement membrane. Concentric layers of outer root sheath, inner root sheath, and hair shaft can be seen. Medulla of the hair shaft can be seen in the center

surface of the HF basement membrane (Fig. 1f) (Rahmani et al. 2014). Recent work suggested that dermal sheath cells can replenish lost dermal papilla cells and are hence progenitors of dermal papilla cells in postnatal life (Rahmani et al. 2014). The harmonious epithelial-mesenchymal interaction is indispensable in modulating the

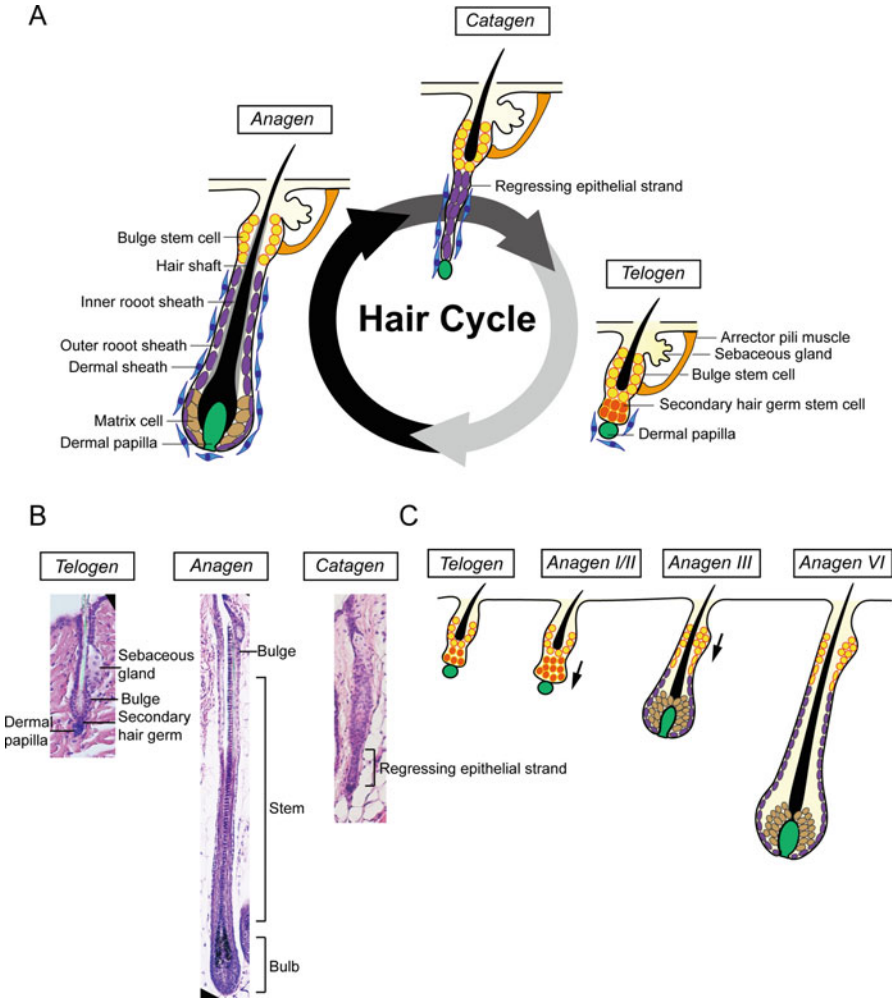


Fig. 2 Structural changes of the HF around the hair cycle and HFSC dynamics during telogen-to-anagen transition. (**a**) Depiction of hair cycles and structural alterations of HFs. During hair cycles, the lower segment below the bulge undergoes dynamic structural alterations. (**b**) Histology of mouse HFs in different hair cycle stages. The lower segment expands and elongates tremendously from telogen to anagen. In telogen, the lower segment regresses toward the bulge. (**c**) HFSC dynamics in telogen-to-anagen transition. In anagen I/II, secondary hair germ HFSCs are activated first to generate the new hair bulb. Bulge HFSCs are activated later in anagen III and contribute to the upper part of the outer root sheath. Shortly after their activation, bulge HFSCs return to an inactivated state and remain quiescent in anagen VI

embryonic HF development and postnatal hair growth (Philpott and Paus 1998; Paus and Cotsarelis 1999; Sennett and Rendl 2012; Millar 2002; Kollar 1970; Dhouailly 1973). In the anagen hair bulb, the dermal papilla exhibits an elongated shape (Fig. 1e). The outer surface of the hair bulb epithelium is a flattened layer of lower proximal cup cells that connect with the ORS cells above (Figs. 1e, 3) (Sequeira and

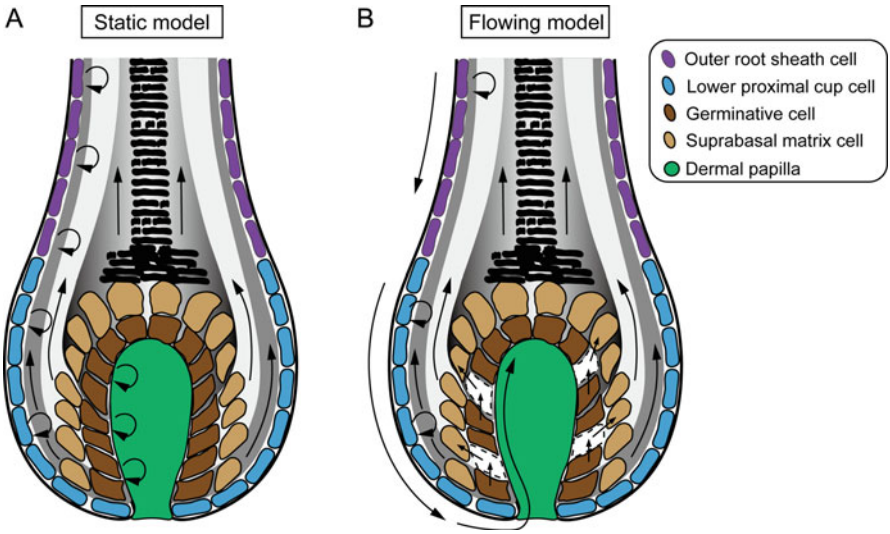


Fig. 3 Models of cell dynamics in the lower segment of an anagen HF. (**a**) Static model. The germinative cells proliferate but are maintained in the basal position. Their progeny move to the suprabasal matrix cell zone and subsequently differentiate into the seven concentric internal layers of the HF. The three distinct progenitors in the outer root sheath, lower proximal cup, and germinative zone do not move to other compartments. They maintain the homeostasis and growth of each compartment, respectively, and their cell fate is restricted to the cell type of their own compartment. (**b**) Flowing model. While germinative cells proliferate to provide new cells for the suprabasal matrix cell zone, some of them can stochastically differentiate and leave the basal layer to the suprabasal matrix cell layer. The germinative cell right below moves up along the basement membrane zone to fill the space previously occupied by the lost germinative cell. Subsequently, lower proximal cup cells move along the basement membrane toward the germinative zone and draw the basal outer root sheath cells down. This creates a net cell flow from the outer root sheath along the basement membrane toward the germinative zone

Nicolas 2012). The inner basal cells abutting dermal papilla across the basement membrane are highly proliferative germinative cells (Figs. 1e, 3). They are among the most actively proliferating cells in the body. It is estimated that cells here divide once a day in human and twice a day in mouse HFs (Chase 1954; Stenn and Paus 2001; Vanscott et al. 1963; Cattaneo et al. 1961). The suprabasal matrix cells are derived from the basal germinative cells and move upward, progressively differentiating to support the continuous elongation of the hair shaft (Figs. 1e, 3) (Sequeira and Nicolas 2012; Yang et al. 2017; Legue and Nicolas 2005).

3 Hair Follicle Development and Patterning

HF development includes three major steps, including induction of HF placode (the initial thickened epithelium for future development of a HF), morphogenesis, and hair differentiation. First, epithelial-mesenchymal interaction allows the formation of hair primordium composed of a hair placode and dermal condensate (Philpott and

Paus 1998; Sennett and Rendl 2012; Millar 2002; Mok et al. 2019). Different ectodermal organs share common features at the molecular level in this stage. Wnt pathway functions as the most upstream signaling to initiate the epithelial-mesenchymal interaction (Millar 2002; Lei et al. 2013). Mesenchymal cell-derived Wnt signaling instructs the epithelial cells to polarize and invaginate into the mesenchymal part (Sennett and Rendl 2012; Millar 2002), leading to formation of a morphologically similar placode during the development of different follicles (hair, feather, and tooth) and different glands (salivary, sebaceous, and mammary).

Second, periodic patterning acts as an epithelial organizer to regulate morphogenesis of complex appendages to generate multiple units in different body regions, allowing the regional specificity to form. For example, when exposed to high mesenchymal bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) secreted by dermal cells in the footpad skin in mice, the epithelial placode develops into the sweat gland, permitting mice to sweat in the feet (Lu et al. 2016). When faced with high sonic hedgehog (SHH) and low BMP signaling, the epithelial placode develops into HFs in the trunk skin of the mice. Another example is that the nipple placode can be converted to form HFs when the BMP signaling is decreased. Mathematical Turing models involving reaction/diffusion of morphogenetic activators and inhibitors have been used to explain the formation of periodic patterning (Maini et al. 2006). In the mouse skin, each hair placode can be an activator to activate itself. It also activates the inhibitors at a longer distance to create spacing between different hair placodes. Wnt and its inhibitor dickkopf-related protein 1 (DKK1) determine the spacing between HFs (Sick et al. 2006), and FGF and BMP spread the ectodysplasin A (EDA) wave to generate the feather spacing pattern during feather development (Inaba et al. 2019; Ho et al. 2019). Periodic patterning also allows the formation of different types of HFs. In mice, hairs at the dorsal, ventral, and facial regions have different sizes and lengths (Lei et al. 2014). The vibrissae on the face are bigger and longer than the dorsal and ventral hairs. Four hair types develop in the mouse dorsum through three waves at the developmental stage (Dry 1926; Sennett and Rendl 2012; Driskell et al. 2009). These include primary HFs (guard, embryonic day 14.5), secondary HFs (auchene and awl, embryonic day 16.5), and tertiary HFs (zigzag, embryonic day 18.5) (Driskell et al. 2009). The primary HF formation is mainly controlled by signaling of FGF20, Wnt10b, EDA/EDAR, etc., whereas the secondary and tertiary HF development is regulated by signaling of EDAR/Troy, Sox18, Noggin/Lef1, etc.

Third, the morphogen signaling cascades induce hair placode cells to differentiate into different cellular components in the HF. Downstream to Wnt signaling, the EDA→EDAR→EDARADD→NF-kB→SHH→cyclin D1 signaling cascade promotes the development of the hair placode into the hair germ (Rishikaysh et al. 2014). The hair germ then develops into a complete HF with sophisticatedly ordered structures (Lei et al. 2017). Molecular signaling pathways including BMP, EDA, FGF, Noggin, Notch, PDGF, SHH, Wnt, etc. are required for these differentiation processes (Chueh et al. 2013). The fully developed HF contains seven internal concentric layers that are derived from the spatially arranged germinative progenitors surrounding the dermal papilla (Yang et al. 2017).

4 HFSCs and Their Activation During Telogen-to-Anagen Transition

Hair regeneration from telogen to anagen relies on the activation of HFSCs (Fig. 2a–c) (Cotsarelis et al. 1990; Greco et al. 2009; Hsu et al. 2011). HFSCs were first identified as slow-cycling cells in the bulge, a localized thickened epithelial structure encircling the hair shaft at the lowermost part of the isthmus, and bulge activation theory was postulated to explain how bulge HFSCs regenerate the lower segment of anagen HFs (Cotsarelis et al. 1990). The structure of the bulge was initially identified in 1876 by Unna who postulated that bulge was the area where the club hair continued to grow ("Haarbeet"; "hair bed") (Ackerman et al. 1993; Unna 1876). This structure was renamed by Stöhr in 1903 as "Wulst" ("bulge") (Ackerman et al. 1993; Stöhr 1903). Recently, cells in the secondary hair germ were identified as another group of stem cells (SCs) in the telogen HFs (Greco et al. 2009; Hsu et al. 2011).

According to the activation dynamics, tissue SCs can be divided into two categories: a less active population of quiescent SCs and a more active population of primed SCs (Potten et al. 1997; Wilson et al. 2008; Li and Clevers 2010). In telogen HFs, two functionally distinct SC populations, quiescent HFSCs and primed HFSCs, are housed in the bulge and secondary hair germ, respectively (Fig. 2a) (Cotsarelis et al. 1990; Greco et al. 2009; Blanpain et al. 2004; Ito et al. 2005; Hsu et al. 2014b; Jaks et al. 2008). Primed HFSCs and quiescent HFSCs are sequentially activated during telogen-to-anagen transition to regenerate the lower transient segment of an anagen HF (Fig. 2c) (Chase 1954; Chase et al. 1951; Greco et al. 2009; Hsu et al. 2011).

Based on the cell dynamics and morphological alterations, anagen is divided into six stages, from anagen I to VI (Fig. 2c) (Muller-Rover et al. 2001; Chase et al. 1951). In anagen I and II, the primed SCs are activated first to produce transit-amplifying cells that contribute to the formation of the initial hair bulb and differentiate into most of the inner layer cells of the transient lower segment (Chase et al. 1951; Legue and Nicolas 2005; Greco et al. 2009; Hsu et al. 2011; Rompolas et al. 2012). Quiescent HFSCs in the bulge are activated later in anagen III, and their descendants regenerate the uppermost ORS of the HF lower segment located right below the bulge (Greco et al. 2009; Hsu et al. 2014b; Rompolas et al. 2012). In contrast to the extensive division of primed HFSCs, bulge HFSCs are activated only temporarily with relatively limited proliferation, rendering them label-retaining (Muller-Rover et al. 2001; Cotsarelis et al. 1990; Greco et al. 2009; Hsu et al. 2011; Tumbar et al. 2004; Zhang et al. 2009). During telogen-to-anagen transition, there is concomitant hyperplasia of dermal white adipose tissue (DWAT) (Chase et al. 1953; Plikus et al. 2008; Festa et al. 2011). The activation of bulge HFSCs and concurrent hyperplasia of DWAT is signaled by HF transit-amplifying cells (Hsu et al. 2014b; Zhang et al. 2016a; Donati et al. 2014). In anagen III to VI, the anagen HF continues to grow downward and invaginates into DWAT. Finally, the produced hair shaft emerges from the skin surface in anagen VI. The duration of anagen VI varies among HFs, leading to variation of the final length of the produced hair shaft.

The structure of secondary hair germ along with the resident primed HFSCs is lost in anagen when HFs grow to the maximal size (anagen VI). It is replaced by the new hair bulb at the proximal end that is connected with the bulge by ORS cells. The highly proliferative matrix cells in the hair bulb continuously differentiate to generate seven internal concentric layers, while the consumption of suprabasal matrix cells are further replenished by germinative cells in the basal layer (Legue et al. 2010). In the meantime, bulge HFSCs remain quiescent and do not contribute to the hair growth in the full anagen (Greco et al. 2009; Hsu et al. 2011, 2014b).

5 Cell Dynamics and Cell Flow in Anagen Hair Follicle

Through cell labeling and tracing, Nicolas and colleagues proposed that the lower transient segment of anagen HFs is maintained by three lineage-distinct populations of basal progenitor cells in the germinative zone, lower proximal cup, and outer root sheath (Fig. 3a) (Sequeira and Nicolas 2012; Legue and Nicolas 2005). In their model, the lower proximal cup cell progenitors maintain the homeostasis of this compartment surrounding the outer surface of the hair bulb, and the ORS is also maintained by its own progenitors (Fig. 3a). Progenitors in these two compartments are immotile and their fates are restricted to each respective cell type. The germinative cells neighboring the dermal papilla give rise to seven internal concentric cell layers through ordered stereotyped differentiation according to their relative position around the dermal papilla (Chase 1954; Sequeira and Nicolas 2012; Legue and Nicolas 2005). This highly ordered spatial differentiation scheme is maintained by the heterogeneity of individual derma papilla cells via their short-range interaction with opposing germinative cells (Yang et al. 2017). Compared with progenitors of lower proximal cup and ORS, the germinative progenitors are therefore highly plastic.

If each basal germinative cell can maintain uninterrupted anagen growth for 30 years in human scalp, it will require a seemingly unlimited potential of cell division, approximately 10,950 times, for each proliferating germinative cell. Without the presence of SCs or a continuous supply of transit-amplifying cells from HFSCs, how can the cell division of germinative cells persist for such an amazingly long duration? In contrast to the static model of positional persistence of HF basal cells, Barrandon and colleagues proposed “flowing” dynamics (Fig. 3b) (Oshima et al. 2001). Through bulge cell transplantation in vibrissal HFs, they demonstrated a continuous flow of basal epithelial cells, from the bulge through ORS and the lower proximal cup to the germinative zone, to replenish germinative cells. Since cells in the ORS and lower proximal cup are relatively slow-cycling as compared with germinative cells, they might serve as reserve SCs (Huang et al. 2017a, 2019; Tian et al. 2011). Considering the duration of anagen of murine vibrissae for up to a couple of months and human scalp hair for decades (Longest head hair (female) 2004), although considerably slow, such a cell flow consistently supplies new cells to the highly proliferative germinative zone. It also suggests that germinative cells are constantly lost in the anagen phase. Recently, Greco and colleagues employed

intravital multiphoton microscopic imaging in mouse pelage HFs and showed that basal germinative cells are constantly lost through suprabasal differentiation and the lost basal cells are replenished by cells from the lower proximal cup and ORS (Xin et al. 2018). Importantly, this flowing dynamics indicates that not only the bulge HFSCs but also cells in the lower proximal cup and ORS are highly plastic. The observation unveils the concealed inherent multipotency of the HF epithelial cells postulated by Chase that epithelial cells in different compartments of HFs are equipotent for hair shaft production (Chase 1954). They can respond to the cues from a new local environment when they change their position in the HF and adopt a new differentiation program accordingly (Chase 1954; Huang et al. 2017a, 2019).

The "static" and "flowing" models are seemingly contradictory. This is due to the scale of time. Nicolas and colleagues' study reflected short-term cell dynamics, while Barrandon and colleagues' cell transplantation involved a longer tracing strategy (Legue and Nicolas 2005; Legue et al. 2010; Oshima et al. 2001). Intravital multiphoton imaging with a relatively long tracing period demonstrated that cell flow from the ORS or lower proximal cup to the germinative zone is a rather slow movement across several days (Xin et al. 2018). Therefore, these two models are basically complementary for the maintenance of anagen growth. The "static" model represents short-term cell renewal/differentiation by local progenitors in each compartment. The "flowing" model depicts the long-term global cell dynamics of progenitors between each compartment of HFs.

6 Organ Regression in Catagen

From anagen to telogen, catagen is believed to be an apoptosis-driven process (Stenn and Paus 2001; Lindner et al. 1997; Mesa et al. 2015). Accompanied by cell death and possibly also cell rearrangement, the lower transient segment progressively shrinks upward to the bulge during catagen (Fig. 2a, b) (Chase 1954; Dry 1926; Hsu et al. 2011; Lindner et al. 1997; Mesa et al. 2015). The length of telogen HF is markedly reduced compared with an anagen HF. A telogen HF consists of a permanent upper segment and a very short lower transient segment, so-called the secondary hair germ (Fig. 2a) (Paus and Cotsarelis 1999). In the telogen phase, cells in the bulge and the secondary hair germ remain inactivated, showing little, if any, cell division.

Catagen is a transient phase, occupying a short duration for about 1 week in mouse pelage HFs (Chase 1954; Muller-Rover et al. 2001; Dry 1926). The first catagen after HF development marks the beginning of lifelong hair cycling (Chase 1954; Muller-Rover et al. 2001; Dry 1926). More than 60 years ago, Chase called for immediate attention to the regulation of catagen (Chase 1954). Despite the progress in hair research, how catagen is regulated has remained largely unexplored (Chase 1954; Stenn and Paus 2001). There are several basic unanswered questions about catagen. How does it begin? How does it progress? How does it stop?

Catagen can be broadly classified into eight stages from catagen I to VIII by the extent of apoptosis and structural regression (Lindner et al. 1997), but rigorous

criteria for characterizing each stage of catagen are lacking. There is also a lack of molecular markers that can specify the start of catagen and each stage of catagen. Conventionally, halt of cell proliferation in the hair bulb is considered as the beginning of catagen (Chase 1954; Dry 1926; Chase et al. 1951; Lindner et al. 1997). Compared with human scalp hair with the potential of continuing anagen for longer than 30 years (Longest head hair (female) 2004), our body hair and the hair in other animals mostly, if not all, have a relatively short duration of anagen and a constant length of hair shafts. These observations suggest that the anagen duration and catagen progression are tightly regulated, possibly by a clock yet to be identified (Paus et al. 1999). It is intriguing whether catagen is a continuum of anagen or an independent phase of hair growth. If catagen is an independent phase of physiological hair growth, whether catagen is triggered by the termination of anagen due to the lack of instructive signals for hair growth or the initiation of catagen actively terminates anagen growth is a topic awaiting exploration.

Generally, the most significant change of catagen is gradual cell death in the HF transient segment to eliminate cells. Cell death can be achieved through various ways, including programmed apoptosis, necroptosis, pyroptosis, necrosis, autophagy, etc. (Tang et al. 2019; Bai et al. 2015). Due to the emergence of activated caspase 3 and positive TUNEL staining, it is commonly assumed that epithelial cells die by apoptosis in catagen (Lindner et al. 1997). Because caspases also exhibit other non-apoptotic functions (Kreuzaler et al. 2011), whether apoptosis is the only way of cell death here is to be confirmed. p53 does not seem to be essential for cell death in catagen, because p53 null mice can still go through catagen (Botchkarev et al. 2001). Live imaging study unveiled that dying epithelial cells are phagocytosed by adjacent keratinocytes in catagen (Mesa et al. 2015). Compared with interfollicular epithelium that generally extrudes apoptotic cells by direct transepithelial elimination (Murphy et al. 2001), engulfing dead cells by follicular epithelial cells seems to be an evolutional novelty of HFs tailored for its characteristic closed structure.

In parallel with epithelial death and removal, there is evidence that dermal sheath cells also undergo concomitant apoptosis in the proximal end (Rahmani et al. 2014). Lineage tracing demonstrated that some dermal sheath cells with a progenitor property survive catagen and are retained around dermal papilla in telogen (Rahmani et al. 2014). Shrinkage of dermal papilla in catagen might involve detachment of some dermal papilla cells due to structural remodeling, and then the lost cells are replenished by dermal sheath progenitor cells in the new anagen (Rahmani et al. 2014; Tobin et al. 2003). In addition to its essential role in promoting anagen entry and maintaining anagen progression through epithelial-mesenchymal interaction (Oshimori and Fuchs 2012; Enshell-Seijffers et al. 2010; Myung et al. 2013), the dermal papilla also promotes catagen progression by inducing epithelial cell death through TGF-β1 signaling (Mesa et al. 2015; Foitzik et al. 2000). Notably, not only cells adjacent to the dermal papilla but also distal epithelial cells away from the dermal papilla undergo apoptosis in catagen (Lindner et al. 1997). We speculate that signals from dermal sheath cells or the lower segment epithelial cells might also regulate cell death during HF regression.

7 HFSC Niche and the Regulation of HFSC Activity by the Niche

First introduced by Schofield (Schofield 1978), the concept of a SC niche was traditionally considered as a specialized microenvironment where SCs are located. The SC niche not only emits signals to nourish or maintain SCs but also filters extrinsic stimuli to prevent apoptosis, senescence, or uncontrolled proliferation/differentiation of SCs. Similar to SCs of other organs, HFSCs reside in a specific microenvironment which is composed of various cell populations (Fig. 4). In addition to the regulation by local niche cells, HFSC activities are also influenced by systemic factors and even the external environment. Due to the complex multi-layered interaction, the SC niche concept can be extended into three levels: micro-niche, macroniche, and meganiche (Fan et al. 2018a). Microniche is the local microenvironment, macroniche refers to the systemic environment (such as circulatory hormones), and meganiche represents the external environment.

Recent advances in hair research have identified a number of cell types, including dermal papilla cells, adipose tissue, lymphatic vessels, nerves, immune cells, etc., to be component cells of the HFSC microniche (Jahoda et al. 1984; Plikus et al. 2008; Festa et al. 2011; Oshimori and Fuchs 2012; Pena-Jimenez et al. 2019; Brownell et al. 2011; Wang et al. 2019; Castellana et al. 2014; Ali et al. 2017; Paus et al. 1994a; Botchkarev et al. 1999; Fan et al. 2018a; Chen et al. 2015; Yu et al. 2018).

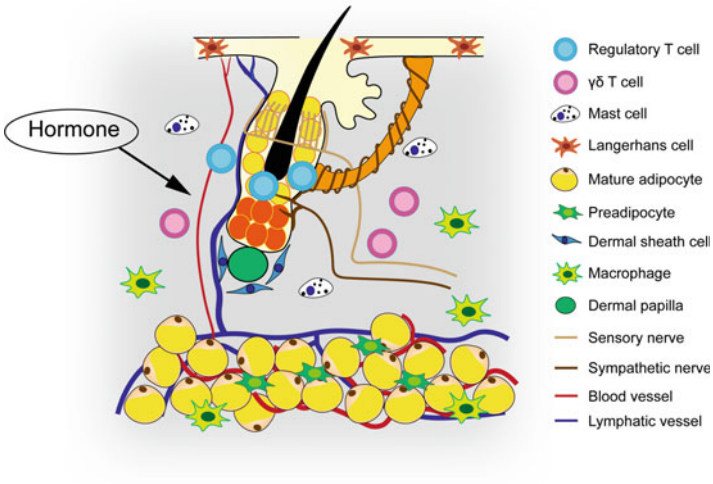


Fig. 4 HFSCs and the niche cells. The HFSC niche is composed of a constellation component cells in direct contact or adjacent to HFSCs. Through direct cell-cell contact and/or paracrine effects, the niche cells not only nourish and maintain HFSCs but also control the activity of HFSCs by providing activating or inhibitory signals. Circulating hormones can act on HFSCs directly or indirectly through niche cells. Since both activating and inhibitory signals can be present at the same time, the decision to remain inactivated or become activated is a readout of the summation of all the signals

The integration of various functionally distinct niche cells confers important properties on HFSCs, especially the ability to respond to changes in local, systemic, and even external environments to adjust their activities. The niche cells can therefore be modularized and individually selected to construct a SC niche. In terms of functional categorization, we divide HFSC niche cells into three groups: signaling, sensing, and message-relaying modules (Chen et al. 2020) (Fig. 5). Signaling modules directly provide activating or inhibitory signals for HFSCs through short-range paracrine effects or by cell-cell contact. Sensing modules detect the local environmental changes, such as injuries and mechanical stimulation, and directly or indirectly activate or suppress HFSCs. Message-relaying modules transmit remote message to the HFSC microenvironment and directly or indirectly regulate HFSC activity. One single niche cell type can be of more than one function.

In the presence of both activating and inhibitory signals, the activity of HFSCs, either quiescence or activation, is the readout of the summation of all the regulatory factors. The HFSC activity is subject to the control of two main opposing signaling pathways: the Wnt/β-catenin and BMP pathways (Plikus et al. 2008, 2011; Plikus 2012). High β-catenin/Wnt signaling promotes HFSC activation and HF growth, while BMP suppresses the HFSC activity (Murray et al. 2012; Plikus et al. 2008,

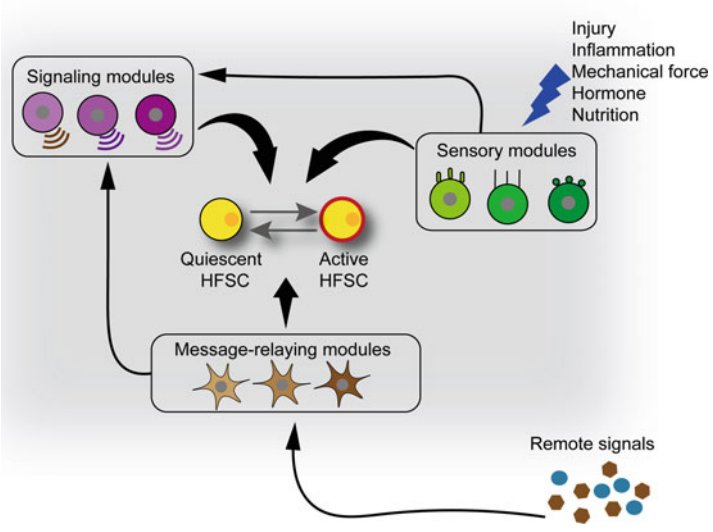


Fig. 5 Categorization of HFSC niche cells according to their functions. In the HFSC niche, the niche cells are divided into different modular groups according to their functions, including signaling modules, sensing modules, and message-relaying modules. These functionally varied modules are assembled into a multifunctional niche. Signaling modules modulate HFSC activity via cell-cell contact and/or a paracrine effect. Sensory modules detect local, systemic, or external environmental changes/cues. Signals from remote cells/tissues can be transmitted to HFSCs by the message-relaying modules, enabling long-range communication of HFSCs with remote cells/tissues. Sensory modules and message-relaying modules can directly control HFSCs or indirectly signal to HFSCs through the signaling modules

2011; Myung et al. 2013; Plikus 2012; Choi et al. 2013). Other signals, such as TGF-β2, oncostatin M, and Foxp1 signaling pathways, are also involved in hair cycle regulation (Oshimori and Fuchs 2012; Wang et al. 2019; Plikus 2012; Leishman et al. 2013). Cues that can tilt the balance of activating and inhibitory signals can modulate HFSC activity, thereby either suppressing or promoting hair regeneration from telogen (Chen et al. 2016; Murray et al. 2012; Oshimori and Fuchs 2012; Wang et al. 2017a, 2019).

8 Dermal Papilla and Dermal Sheath

Dermal papilla, the major follicular mesenchymal cells, is in direct contact with primed HFSCs in the secondary hair germ. Epithelial-mesenchymal interaction between the follicular epithelial cells and mesenchyme is essential for both embryonic HF development and HF cycling (Sennett and Rendl 2012; Rompolas et al. 2012; Mesa et al. 2015; Yu et al. 2018). During telogen-to-anagen transition, dermal papilla cells activate HFSCs by releasing activating ligands such as TGF-β2 and FGF-7 (Greco et al. 2009; Oshimori and Fuchs 2012), while epithelial signals also critically contribute to anagen entry (Myung et al. 2013). After HFs reach mature anagen, dermal papilla not only promotes germinative cell proliferation but also instructs the spatially ordered differentiation of their progeny in the hair matrix into seven distinct concentric layers to support continuous elongation of the hair shaft (Yang et al. 2017). Dermal papilla is a key controller of the size of the hair shaft and hair types (Huang et al. 2013; Chi et al. 2013; Elliott et al. 1999). Experimental reduction of dermal papilla cell numbers leads to miniaturization of the hair shaft and corresponding changes in hair types (Chi et al. 2013). Although the signaling process of how dermal papilla regulates the hair size and guides spatially distinct differentiation of germinative cell progeny is unclear, suppression of Wnt/β-catenin signaling in dermal papilla halts normal anagen and induces premature catagen entry, demonstrating an essential role of Wnt/β-catenin signaling in dermal papilla cells to maintain anagen hair growth (Enshell-Seijffers et al. 2010). Additionally, dermal papilla is also required for the controlled cell death/apoptosis of the regressing epithelial strands in catagen (Mesa et al. 2015). During HF repair from chemotherapy or radiotherapy injury, dermal papilla might also instruct the regenerative process to bypass premature catagen entry (Huang et al. 2017a, 2019) (please refer to the later subchapter where this process is described in more detail). In summary, signals from dermal papilla are required for HFSC activation, anagen progression/maintenance, hair shaft formation, and catagen progression.

The dermal sheath cells are another group of mesenchymal fibroblasts forming a sheet enveloping the outer surface of the cylindrical HF basement membrane. Their spindle-shaped cell body along with their intracellular smooth muscle actin fibers parallelly and horizontally surrounds the HF (Rahmani et al. 2014; Heitman et al. 2020). In anagen, their contractile property might help to maintain the cylindrical shape of HFs. When the epithelial strand regresses in catagen, their centripetal contractile force contributes to the upward movement of the hair shaft and dermal

papilla, relocating dermal papilla to the HFSC niche (Heitman et al. 2020). Lineage tracing discovered that part of dermal sheath cells possess the ability of self-renewal and can provide new cells for dermal papilla and new dermal sheath during telogen-to-anagen transition (Rahmani et al. 2014). Similar to dermal papilla cells, isolated dermal sheath cells are able to induce neogenesis of HFs when combined with keratinocytes (McElwee et al. 2003; Horne and Jahoda 1992), highlighting their capability to reprogram interfollicular keratinocytes toward follicular fates. Whether dermal sheath cells regulate HFSC activity during telogen-to-anagen transition remains to be explored.

9 Adipose Tissue

In addition to being a passive depot of fat, adipose tissue also has other important non-metabolic functions (Guerrero-Juarez and Plikus 2018). In face of cutaneous bacterial infection, local adipocytes quickly undergo reactive adipogenesis to increase antimicrobial peptide secretion to restrict bacterial invasion (Zhang et al. 2015). In close contact with HFSCs, DWAT is an important regulator of HFSC activity (Zwick et al. 2018). As integral parts of the skin, HFs and DWAT signal to and regulate each other. As hair cycles, the thickness of DWAT also shows parallel oscillation, thinner in telogen and thicker in anagen (Chase et al. 1953; Festa et al. 2011; Hansen et al. 1984; Nicu et al. 2019). Proliferation and differentiation of preadipocytes and hypertrophy of adipocytes lead to expansion of the DWAT in early anagen (Festa et al. 2011; Rodeheffer et al. 2008). This process of increased adipogenesis is dependent on Wnt/β-catenin and SHH signaling from the growing HFs (Donati et al. 2014; Zhang et al. 2016b). The shrinkage of DWAT during anagen-to-catagen transition does not involve apoptosis of mature adipocytes (Festa et al. 2011) and is mediated by a lipolytic change and dedifferentiation of mature adipocytes (Nicu et al. 2019; Zhang et al. 2019).

In adipocytes, cyclic expression of BMPs 2/4, which are out of phase with the intrafollicular Wnt/β-catenin cycle, suppresses the activity of HFSC and divides the traditional hair cycle into five functional phases: propagatory anagen, autonomous anagen, catagen, refractory telogen, and competent telogen (Plikus et al. 2008). Increased release of BMPs 2/4 from adipocytes shortly after HFSC activation thus prevents overactivation of HFSCs. Following this idea, more dermal inhibitors, including DKK1 and secreted frizzled-related protein 4 (SFRP-4), which are both expressed in autonomous anagen and refractory telogen were identified to suppress the activity of HFSCs (Plikus et al. 2011). Other than inhibitors, DWAT also produces activators, such as follistatin, to stimulate hair wave propagation during propagatory anagen (Chen et al. 2014). Platelet-derived growth factor-α (PDGFA) released by the dermal adipocyte precursor cells also promotes anagen entry (Festa et al. 2011). As skin ages, hair regeneration cycles are retarded due to overexpression of all the dermal inhibitors (BMP2, DKK1, Sfrp4, etc.) with concomitant down-regulation of activators (follistatin, etc.) (Chen et al. 2014). The reciprocal signaling and interaction between HFs and DWAT demonstrate the interdependent relationship

between HFSCs and its niche cells to sustain appropriate cutaneous tissue dynamics. Age-related changes in niche cells can compromise such dynamics.

10 Sensory Nerves, Sympathetic Nerves, and Modulation of HFSC Activity Via the ipRGC-SCN-Sympathetic Nervous Circuit by External Light

Dense sensory innervation capacitates HFs to conduct its unique mechanosensory functions (Cheng et al. 2018; Botchkarev et al. 1997). Sensory nerves form a delicate network around the upper bulge and the sensory piloneural niche (Fig. 4) (Cheng et al. 2018; Botchkarev et al. 1997). Sensory nerves signal upper bulge HFSCs via the release of SHH ligands (Brownell et al. 2011). Upregulated hedgehog signaling, as shown by the increase of downstream transcriptional factor Gli1, enables the upper bulge HFSCs to respond to insults for reepithelialization (Brownell et al. 2011). Due to the limited range of action of SHH ligands, sensory nerves do not significantly influence HFSCs at the lower bulge and secondary hair germ for hair regeneration (Brownell et al. 2011).

Piloerection of HFs, or goose bumps, depends on the incorporation of sympathetic nerves and arrector pili muscle during development. The bulge is the place where arrector pili muscle attaches to the HF (Fig. 1c). In human, not all HFs are of a piloerectile function due to the lack of arrector pili muscles in some body regions, such as HFs in the beard and pubic area (Ackerman et al. 1993). Sympathetic nerves not only densely surround arrector pili muscle for the induction of piloerection but also loop around HFSCs (Fig. 4) (Botchkarev et al. 1997; Fan et al. 2018a). The possible stimulation of HFSC activity by sympathetic nerves is suggested by clinical cases showing hair overgrowth or hypertrichosis in the form of "hemitrichosis" (hair overgrowth on one side of the body) due to thoracic surgery-induced sympathetic nerve hyperactivity (Mendez-Ferrer et al. 2008). Animal and in vitro experiments suggested that sympathetic nerves might accelerate anagen progression after initial anagen entry (Botchkarev et al. 1997).

Circadian rhythms which oscillate with the external light cycle affect most physiological processes, including HFs (Plikus et al. 2013). The major circadian rhythm genes, including Clock, Bmal1, and Period1, have been shown to regulate hair cycle and mitotic rates of anagen HFs (Plikus et al. 2013; Lin et al. 2009; Al-Nuaimi et al. 2014). Without resetting the central circadian rhythm, external light is able to modulate HFSCs in other ways. Low-level light irradiation, especially long-wavelength light such as red light, to skin can directly promote anagen entry (Sheen et al. 2015; Fushimi et al. 2011; Mai-Yi Fan et al. 2018). This is possibly mediated by a photomodulation effect through mitochondria that enhances follicular epithelial-mesenchymal interaction (Sheen et al. 2015). High-intensity laser otherwise promotes anagen entry by creating controlled thermal injuries to skin (Wu et al. 2015).

External light, preferentially blue light, can also indirectly activate HFSC through the eyes (Fig. 6) (Fan et al. 2018a; Sheen et al. 2015; Mai-Yi Fan et al. 2018). Light is

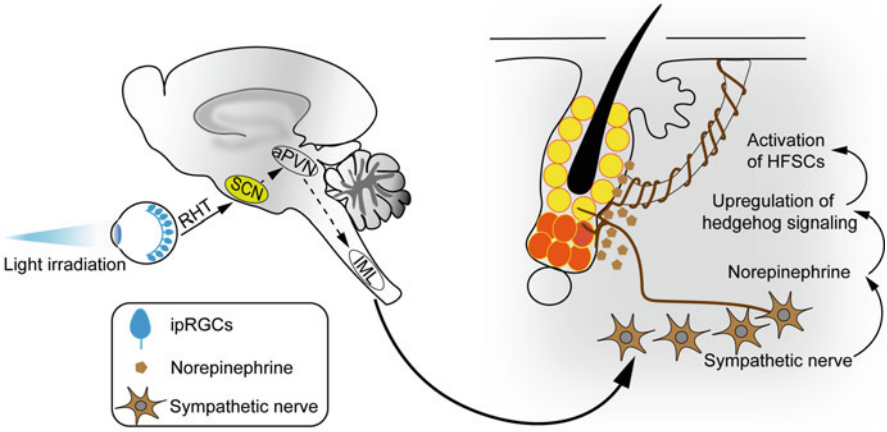


Fig. 6 Detection of external light signals through an ipRGC-SCN-sympathetic nervous circuit. Sympathetic nerves innervate HFSCs before they loop around the arrector pili muscle. They are of a message-relaying function in the HFSC niche and create a gateway for the communication between internal HFSCs and the external environment. Intense light irradiation to eyes is first detected by ipRGCs through the atypical photoreceptor melanopsin in the retina. Activated ipRGCs immediately activate the systemic sympathetic nervous system via SCN. Norepinephrine released from cutaneous sympathetic nerves promotes HFSC activation by upregulating hedgehog signaling. *aPVN* autonomic neurons of the paraventricular nucleus, *IML* intermediolateral cell column, *RHT* retinohypothalamic tract, *SCN* suprachiasmatic nucleus

detected by intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina (Fan et al. 2018a). Subsequently, the light signals are transmitted via ocular nerves to the suprachiasmatic nucleus (SCN), which elevates the systemic sympathetic tone. In skin, increased norepinephrine release from sympathetic nerves activates hedgehog signaling in HFSCs, leading to premature HFSC activation and hair regeneration (Fan et al. 2018a). Therefore, sympathetic nerves are of dual functions in the HFSC niche. First, they induce arrector pili muscle contraction for piloerection. Second, they are message-relaying modules of the HFSC niche, which transmit external light signals to HFSCs via an ipRGC-SCN-sympathetic nervous circuit, enabling HFSCs to detect the changes of the external environment. For clinical application, agonists to adrenergic receptors of HFSCs can be of potential for the treatment of alopecia (Fan et al. 2018a).

11 Immune Cells and Immunity-Induced Hair Loss

The immune system is an important regulator of HFSC activity (Fig. 4) (Castellana et al. 2014; Ali et al. 2017; Paus et al. 1994a; Chen et al. 2015). Proper HF cycling is strongly dependent on the homeostasis of its immune cells as well as maintenance of its immune privilege (Paus et al. 2003).

In the physiological condition, skin-resident forkhead box P3 (FOXP3)-expressing regulatory T cells (Tregs) are located in the follicular epithelium in close contact with HFSCs (Ali et al. 2017). They promote the proliferation and differentiation of HFSCs through the Jag1-Notch signaling pathway (Ali et al. 2017). Additionally, activated γδ T cells fasten wound healing by stimulating interfollicular epidermal basal cells and also promote HFSC activation for hair regrowth (Lee et al. 2017; Jameson and Havran 2007).

Disruption of its immune privilege can put HF under immune attacks (Paus et al. 2003; Gilhar et al. 2007). Alopecia areata, or so-called spot baldness, is the most common immunity-mediated hair loss due to the collapse of follicular immune privilege, leading to emergence of autoreactive T cells in the HFSC niche. It is a reversible non-scarring alopecia in which HFSCs are preserved despite the inflammatory attack (Strazzulla et al. 2018). The severity of alopecia areata can vary from a single patch of hair loss on the scalp to universal loss of hair over the entire skin surface (Strazzulla et al. 2018). Although the exact etiopathogenesis of alopecia areata is not fully understood, it is commonly accepted that, due to collapsed immune privilege, increased presentation of autoantigens to surveying T cells that recognize HF antigens leads to autoimmune responses against HF cells (Gilhar et al. 2007; Strazzulla et al. 2018; Wang and McElwee 2011). The inflammatory response preferentially attacks the lower transient segment of anagen HFs, thus arresting hair growth with premature telogen induction (Strazzulla et al. 2018). If the inflammation continues, HFSC activity is suppressed and HFs are arrested in telogen. Removal of these pathogenic T cells from the HFSC niche by immunosuppression, such as local or systemic steroids, or immunomodulation, such as topical contact sensitizers, can reactivate HFSCs to restore hair growth (Alkhalifah et al. 2010; Cotellessa et al. 2001).

A population of $CD8^+/NKG2D^+$ T cells is necessary and sufficient for alopecia areata (Xing et al. 2014; Petukhova et al. 2010; Betz et al. 2015). Since JAK-STAT signaling is essential for $CD8^+/NKG2D^+$ T cell functions, small-molecule inhibitors, such as tofacitinib and ruxolitinib, that can effectively block JAK-STAT signaling not only reverse alopecia areata in the mouse model but also restore hair growth in alopecia areata patients (Wang et al. 2018). These observations show that influx of autoreacting T cells into the HFSC niche can be detrimental to normal hair growth.

Mast cells are also present in the perifollicular space (Kumamoto et al. 2003). Mast cells degranulate during the transition from late telogen to early anagen and the transition from late anagen to early catagen (Paus et al. 1994a; Maurer et al. 1995). Molecules, such as histamine and serotonin, released from mast cells by degranulation might contribute to HF turnover (Maurer et al. 1997).

12 Macrophages and Hair Regeneration in Response to Injury and Mechanical Force

Another group of immune cells present around HFs are macrophages which have been shown to modulate hair cycles (Castellana et al. 2014; Paus et al. 1998). Macrophages in the skin are heterogeneous and can have opposite effects on

HFSCs. The number of skin-resident $CD11b^{+}F4/80^{+}Gr1^{-}$ macrophages oscillates during hair cycles. Their number decreases shortly before the onset of anagen due to apoptosis which increases their release of Wnt7b and Wnt10a, thus facilitating HFSC activation and differentiation (Castellana et al. 2014). In contrast, $TREM2^{+}$ dermal macrophages suppress hair growth through a paracrine effect of oncostatin M (Wang et al. 2019). It was first found that inhibition of JAK-STAT signaling also promotes hair growth by breaking HFSC quiescence (Harel et al. 2015). Oncostatin M produced by $TREM2^{+}$ macrophages acts upstream to enhance JAK-STAT signaling, thus inhibiting HFSC activation (Wang et al. 2019). Ablation of oncostatin M-producing $TREM2^{+}$ macrophages halts the suppression and accelerates anagen entry (Wang et al. 2019).

Macrophages also enable HFSCs to sense and respond to injuries and mechanical cues. Classical studies showed that hair plucking, a minor intrafollicular trauma, is a potent HFSC activator (Silver and Chase 1970; Collins 1918). It involves a two-step process: early apoptotic cell death of bulge HFSCs, followed by activation and proliferation of secondary hair germ HFSCs (Ito et al. 2002). Recent work demonstrated that HFSC activation can occur through a threshold-dependent collective decision-making process, named quorum sensing (Chen et al. 2015). When hair plucking is properly arranged, more than five times of neighboring unplucked telogen HFs can be activated to enter a new anagen. This all-or-none-threshold-dependent regeneration process is mediated through an immune response cascade. First, injured HFs release CCL2 that recruits TNF-α-secreting macrophages, which accumulate and signal to both plucked and unplucked HFs (Chen et al. 2015). The released TNF-α activates HFSCs through AKT-dependent nuclear β-catenin accumulation (Wang et al. 2017b). This quorum sensing behavior made possible by macrophages provides a novel idea that SCs at a population level decide whether to neglect a stimulus or to react to a stimulus with a full-scale cooperative regenerative response depending on whether the stimulus has reached a threshold.

When skin is wounded, macrophages can also be activated through the apoptosis signal-regulating kinase 1 (ASK1) (Osaka et al. 2007). Specifically, $CX_3CR1^{high}CCR2^{+}$ macrophages expressing TNF-α and TGF-β1 are critical in wound-induced hair growth (Rahmani et al. 2018). TNF-α-secreting macrophages activate HFSCs via the TNF-α/p-AKT/p-β-catenin-Ser552 signaling axis, promoting anagen entry in the skin adjacent to the wounds (Wu et al. 2015; Osaka et al. 2007; Rahmani et al. 2018).

Interestingly, macrophages also capacitate HFSCs to sense and respond to mechanical force in a quorum-sensing manner. By utilizing a specially designed skin-stretching device, Chu and colleagues showed that HFSCs within a skin field can be activated when proper strain of an appropriate duration of stretching is achieved (Chu et al. 2019). If the combination of strain force and stretching duration does not reach a certain threshold, all HFSCs in the stretched skin remain quiescent. Proper stretching polarizes cutaneous macrophages into a M2 phenotype (Chu et al. 2019). M2 macrophages are pro-regenerative and stimulate HFSCs for hair regeneration via the production of growth factors, such as IGF and HGF. Therefore, macrophages are key sensing cells that enable HFSCs to respond to intrafollicular

and extrafollicular injuries and to mechanical stretch to start a new round of hair growth. Targeting macrophages can be a potential treatment to promote hair growth.

13 Hormones

Clinically, androgen is the most important hormone that regulates hair growth. Androgen paradoxically affects hair growth (Thornton et al. 1993; Randall et al. 2000). After puberty, elevation of circulating androgen can stimulate fine vellus hair to grow into thick terminal hair in the axillary, pubic, and beard HFs (Marshall and Tanner 1969, 1970). On the contrary, it also induces androgenetic alopecia, commonly named male pattern hair loss, with progressive HF miniaturization in the scalp of genetically predisposed patients (Nyholt et al. 2003). Hyperandrogenism in females can lead to hirsutism characterized by excessive male pattern hair growth and even with male pattern hair loss (Azziz 2003). In HFs, intracellular androgen receptors are mainly located in the dermal papilla cells (Hibberts et al. 1998; Ando et al. 1999). Keratinocytes neither express androgen receptors nor show significant androgen receptor-dependent signaling activation (Choudhry et al. 1992; Lai et al. 2012). Therefore, niche component dermal papilla cells, but not HFSCs, are the primary cells that sense and respond to circulating androgen.

Androgenetic alopecia can be considered as a form of premature HF aging, featured by organ atrophy, prolonged telogen, and even loss of the entire HFs (Courtois et al. 1995; Matsumura et al. 2016). In the balding scalp, dermal papilla cells incorrectly exhibit a high activity of type II 5α-reductase, an enzyme that is normally highly expressed in the prostate (Ando et al. 1999). Type II 5α-reductase converts testosterone into dihydrotestosterone, leading to local accumulation of dihydrotestosterone (Hibberts et al. 1998). Sustained dihydrotestosterone stimulation to dermal papilla cells induces senescent changes and disrupts their molecular signature and functions (Thornton et al. 1993; Randall et al. 2000, 1996; Bahta et al. 2008). The resultant shrinkage of dermal papilla and its altered signaling lead to HF atrophy, shortened anagen, and prolonged telogen.

The balding dermal papilla cells not only lose the ability to promote HFSC proliferation but also produce inhibitory factors that suppress HFSCs and compromise keratinocyte proliferation (Huang et al. 2017b; Kwack et al. 2008, 2012; Inui et al. 2003). For example, the negative Wnt regulator of DKK1 and catagen-promoting TGF-β1 are overexpressed by balding dermal papilla cells (Mesa et al. 2015; Kwack et al. 2008; Inui et al. 2003; Paus et al. 1997). Balding dermal papilla cells also produce higher levels of inflammatory cytokines such as IL-6 (Huang et al. 2017b; Kwack et al. 2012; Turksen et al. 1992), which can inhibit anagen entry and interfere with normal anagen (Huang et al. 2017b; Kwack et al. 2012; Turksen et al. 1992).

The first FDA-approved medication for treating androgenetic alopecia is minoxidil (Rossi et al. 2012; De Villez 1985), which was initially designed to target potassium channel for the treatment of hypertension (Zappacosta 1980). Although the mechanisms are not well understood, minoxidil might promote hair growth through its effects on perifollicular vessels or follicular potassium channels

(Headington 1987). Because minoxidil does not block the basic etiopathogenesis of androgenetic alopecia, long-term use of minoxidil fails to halt the gradual organ atrophy.

Later development of inhibitors to local dihydrotestosterone production in the balding region provides the most effective treatment for androgenetic alopecia. Finasteride and dutasteride inhibit 5α-reductase activity with varied specificity and potency (McConnell et al. 1992; Bramson et al. 1997). Finasteride is a type II 5α-reductase inhibitor, while dutasteride is an inhibitor of both type I and type II 5α-reductases. Long-term suppression of dihydrotestosterone production by oral finasteride and dutasteride increases hair growth in androgenetic alopecia patients and partially reverses the alopecic change (Kaufman et al. 1998; Olsen et al. 2006).

14 Blood and Lymphatic Vessels

Although HFs depend on perivascular vessels for the supply of oxygen and nutrients, whether intradermal vasculature regulates HFSC activity remained elusive. Recently, direct cross talks between vascular endothelial cells and HFSCs were revealed (Li et al. 2019). While HF epithelial cells regulate the perifollicular vascular plexus remodeling during hair cycles, skin vasculature suppresses HFSC activation by producing BMP4 (Li et al. 2019). Therefore, vascular endothelial cells help to maintain the quiescence of HFSCs and restrain the progression from telogen to anagen (Li et al. 2019). In addition to blood vessels, there are also cross talks between the perifollicular lymphatic vessels and HFSCs (Pena-Jimenez et al. 2019; Gur-Cohen et al. 2019). As hair cycles, HFSCs instruct the remodeling of perivascular lymphatic vessels through the expression of Angptl7 and Angptl4. Perturbing lymphatic vessels lead to alteration of HFSC activation and hair cycles (Pena-Jimenez et al. 2019; Gur-Cohen et al. 2019).

15 Anagen Hair Follicle Repair to Resume Disrupted Anagen After Follicular Damage

In addition to physiological telogen-to-anagen regeneration, there is another form of regeneration in response to injuries to anagen HFs, named anagen HF repair (**AHFR**) (Huang et al. 2017a, 2019). AHFR is the regenerative scheme to restore the lost structures to restore anagen growth after HF damage, such as injuries by chemotherapy, radiotherapy, and inflammation (Huang et al. 2019). Compared with telogen-to-anagen regeneration, AHFR is a relatively unexplored regenerative program of HFs.

Due to its highly proliferative nature, anagen HFs are sensitive to genotoxic injury from chemotherapy and radiotherapy. Chemotherapy and radiotherapy induce apoptosis of proliferating cells in the hair bulb, thus impeding hair growth. After such damage, anagen HFs exhibit two regenerative pathways (Huang et al. 2017a; Paus et al. 1994b, 2013; Wang et al. 2017c). When HFs are severely damaged by

higher doses of ionizing radiation and chemotherapeutic agents, HFs enter a dystrophic catagen pathway with premature telogen induction. This resets hair cycle and HFs can only regenerate in the following anagen phase. If HFs are damaged to a lesser extent, HFs initiate a dystrophic anagen pathway in which AHFR replenishes the lost cells and restores the lost structures (Huang et al. 2017a, 2019) (Fig. 7). HFs immediately recommence the same anagen after AHFR without hair cycle resetting.

According to the severity of injuries, HFs are capable of launching two tempospatially distinct regenerative attempts of AHFR in the dystrophic anagen response (Fig. 7) (Huang et al. 2017a). Within 24 h after 2 Gy of ionizing radiation injury, anagen HFs start an early anagen regenerative attempt (Huang et al. 2017a). Bulge HFSCs are not involved in this process. Instead, the basal cells from the lower proximal cup and ORS are quickly mobilized. Their progeny quickly contributes to the regeneration of the damaged germinative zone in the hair matrix and subsequently the affected internal structures of anagen HFs (Fig. 7). Compared with the relatively slow flow of lower proximal cup cells into the germinative zone in the physiological state (Xin et al. 2018), the movement of lower proximal cup cells is accelerated in AHFR (Huang et al. 2017a). Using this timely strategy to mobilize local plastic progenitor cells for regeneration, the halted anagen is quickly resumed with minimal hair loss.

In face of more severe damage by 5.5 Gy of ionizing radiation which leads to prolonged and extensive cell death, the early regenerative attempt fails (Huang et al. 2017a). The hair bulb structure is lost, and the HF is shortened into an epithelial strand by remodeling ORS cells at about 72 h (Fig. 7). Structurally, this epithelial strand is still longer than a telogen HF. Surprisingly, its lower tip cells acquire a secondary hair germ HFSC-like property. These primed HFSC-like cells start to proliferate and promptly regenerate most of the lower segment structures including the hair bulb in a way similar to telogen-to-anagen regeneration (Fig. 2). Bulge HFSCs are activated later and play a relatively minor role, regenerating only part of the upper ORS cells. This late regenerative attempt also helps to bypass hair cycle resetting and the HF restarts the interrupted anagen.

Mechanistically, mTOR signaling is activated in the hair bulb in AHFR (Wang et al. 2017c). Active mTOR signaling reduces cell death and promotes compensatory regenerative proliferation to restore the hair bulb structures. Wnt signaling is not only essential for normal anagen maintenance but also required for successful AHFR (Huang et al. 2017a; Enshell-Seijffers et al. 2010; Myung et al. 2013). Forced activation of Wnt signaling can prevent hair loss from chemotherapy and radiotherapy by facilitating the mobilization of basal progenitor cells of the lower proximal cup into the damaged germinative zone (Huang et al. 2017a). In normal anagen, SHH ligands are constantly produced by matrix cells, and active hedgehog signaling is essential for anagen growth (Hsu et al. 2014b; Zhang et al. 2016a; Paladini et al. 2005). Hedgehog signaling has also been shown to be suppressed in HFs after chemotherapy and radiotherapy (Huang et al. 2017a; Xie et al. 2015). Because suppression of hedgehog signaling may contribute to dystrophic changes of HFs, modifying hedgehog signaling can also be a potential strategy to prevent hair loss by promoting AHFR.

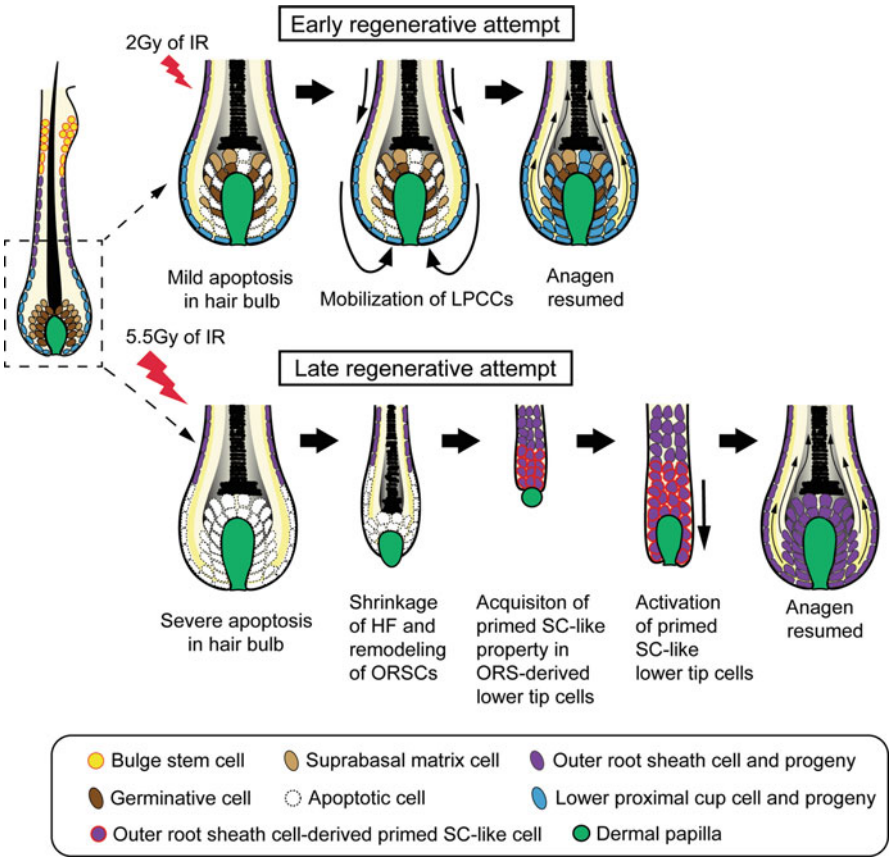


Fig. 7 Cell dynamics of anagen hair follicle repair. Anagen HFs can choreograph tempospatially distinct regenerative schemes according to the severity of injuries. Following a minor injury of 2 Gy of ionizing radiation, anagen HFs quickly mobilize lower proximal cup cells to replenish the lost germinative cells. The progenies of basal lower proximal cup cells are highly plastic and regenerate the internal HF structures. Facing a severe injury by 5.5 Gy of ionizing radiation, the early regenerative attempts fail to repair the damaged hair bulb due to more extensive cell death. Anagen HFs then stage a late regenerative attempt. The outer root sheath is first remodeled into a short epithelial strand, and the cells in the lower tip contacting the dermal papilla acquire a secondary hair germ HFSC-like property. These new progenitor cells regenerate most parts of the lower segment to recover the anagen

16 Conclusions

The discovery of slow-cycling bulge HFSCs and the more active secondary hair germ HFSCs has hastened the advance in the understanding of hair growth regulation (Cotsarelis et al. 1990; Greco et al. 2009; Jaks et al. 2008). In addition to the intrinsic regulatory networks of HFSCs, various niche component cells have been

identified. Their roles in hair growth can be categorized according to their distinct functions, such as signaling, sensing, and message-relaying. The multifunctional niche enables HFSCs to interpret the local, systemic, and external environmental cues to adapt their activity to meet physiological needs. Understanding how the niche regulatory networks are altered in diseased HFs can therefore provide novel insight for the development of new treatment strategies. Recent discovery of the intrinsic regenerative attempts of AHFR in response to HF damage during anagen demonstrated the plasticity of nonphysiological precursor cells in the lower transient segment of anagen HFs. Aiming at the molecular regulation of AHFR can accelerate structural recovery of damaged anagen HFs to prevent hair loss from radiotherapy and chemotherapy.

Acknowledgments This work was supported by Taiwan Bio-Development Foundation (TBF) (to SJL), Taiwan Ministry of Science and Technology (MOST107-2314-B-002-064-MY3; 108-2811-B-002-583), National Taiwan University Hospital (UN108-029, 109S4567, 108-T17, 109-T17), and Taiwan National Health Research Institutes (NHRI-EX108-10811EI, NHRI-EX109-10811EI).

References

Abaci HE et al (2018) Tissue engineering of human hair follicles using a biomimetic developmental approach. Nat Commun 9(1):5301

Ackerman AB, De Viragh PA, Chongchitnant N (1993) Anatomic, histologic and biologic aspects of hair follicles and hairs. In: Neoplasms with follicular differentiation. Lea & Febiger, Philadelphia/London, pp 35–102

Ali N et al (2017) Regulatory T cells in skin facilitate epithelial stem cell differentiation. Cell 169(6):1119–1129. e11

Alkhalifah A et al (2010) Alopecia areata update: part II. Treatment. J Am Acad Dermatol 62(2):191–202. quiz 203-4

Al-Nuaimi Y et al (2014) A meeting of two chronobiological systems: circadian proteins Period1 and BMAL1 modulate the human hair cycle clock. J Invest Dermatol 134(3):610–619

Ando Y et al (1999) Expression of mRNA for androgen receptor, 5 alpha-reductase and 17 beta-hydroxysteroid dehydrogenase in human dermal papilla cells. Br J Dermatol 141(5):840–845

Azziz R (2003) The evaluation and management of hirsutism. Obstet Gynecol 101(5 Pt 1):995–1007

Bahta AW et al (2008) Premature senescence of balding dermal papilla cells in vitro is associated with p16(INK4a) expression. J Invest Dermatol 128(5):1088–1094

Bai X et al (2015) Roles of GasderminA3 in Catagen-Telogen transition during hair cycling. J Invest Dermatol 135(9):2162–2172

Betz RC et al (2015) Genome-wide meta-analysis in alopecia areata resolves HLA associations and reveals two new susceptibility loci. Nat Commun 6:5966

Blanpain C et al (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118(5):635–648

Botchkarev VA et al (1997) Hair cycle-dependent plasticity of skin and hair follicle innervation in normal murine skin. J Comp Neurol 386(3):379–395

Botchkarev VA et al (1999) Hair cycle-dependent changes in adrenergic skin innervation, and hair growth modulation by adrenergic drugs. J Invest Dermatol 113(6):878–887

Botchkarev VA et al (2001) p53 involvement in the control of murine hair follicle regression. Am J Pathol 158(6):1913–1919

Bramson HN et al (1997) Unique preclinical characteristics of GG745, a potent dual inhibitor of 5AR. J Pharmacol Exp Ther 282(3):1496–1502
Brownell I et al (2011) Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell 8(5):552–565
Castellana D, Paus R, Perez-Moreno M (2014) Macrophages contribute to the cyclic activation of adult hair follicle stem cells. PLoS Biol 12(12):e1002002
Cattaneo SM, Quastler H, Sherman FG (1961) Proliferative cycle in the growing hair follicle of the mouse. Nature 190:923–924
Chase HB (1954) Growth of the hair. Physiol Rev 34(1):113–126
Chase HB, Rauch R, Smith VW (1951) Critical stages of hair development and pigmentation in the mouse. Physiol Zool 24(1):1–8
Chase HB, Montagna W, Malone JD (1953) Changes in the skin in relation to the hair growth cycle. Anat Rec 116(1):75–81
Chen CC et al (2014) Regenerative hair waves in aging mice and extra-follicular modulators follistatin, dkk1, and sfrp4. J Invest Dermatol 134(8):2086–2096
Chen CC et al (2015) Organ-level quorum sensing directs regeneration in hair stem cell populations. Cell 161(2):277–290
Chen CC et al (2016) The Modulatable stem cell niche: tissue interactions during hair and feather follicle regeneration. J Mol Biol 428(7):1423–1440
Chen CL et al (2020.. (in press)) Functional complexity of hair follicle stem cell niche and therapeutic targeting of niche dysfunction for hair regeneration. J Biomed Sci 27:43
Cheng CC et al (2018) Hair follicle epidermal stem cells define a niche for tactile sensation. Elife 7
Chi W, Wu E, Morgan BA (2013) Dermal papilla cell number specifies hair size, shape and cycling and its reduction causes follicular decline. Development 140(8):1676–1683
Choi YS et al (2013) Distinct functions for Wnt/beta-catenin in hair follicle stem cell proliferation and survival and interfollicular epidermal homeostasis. Cell Stem Cell 13(6):720–733
Choudhry R et al (1992) Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. J Endocrinol 133(3):467–475
Chu S-Y et al (2019) Mechanical stretch induces hair regeneration through the alternative activation of macrophages. Nat Commun 10(1):1524
Chueh SC et al (2013) Therapeutic strategy for hair regeneration: hair cycle activation, niche environment modulation, wound-induced follicle neogenesis, and stem cell engineering. Expert Opin Biol Ther 13(3):377–391
Collins HH (1918) Studies of normal moult and of artificially induced regeneration of pelage in Peromyscus. J Exp Zool 27:27
Cotellessa C et al (2001) The use of topical diphenylcyclopropenone for the treatment of extensive alopecia areata. J Am Acad Dermatol 44(1):73–76
Cotsarelis G, Sun TT, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61(7):1329–1337
Courtois M et al (1995) Ageing and hair cycles. Br J Dermatol 132(1):86–93
De Villez RL (1985) Topical minoxidil therapy in hereditary androgenetic alopecia. Arch Dermatol 121(2):197–202
Dhouailly D (1973) Dermo-epidermal interactions between birds and mammals: differentiation of cutaneous appendages. J Embryol Exp Morphol 30(3):587–603
Donati G et al (2014) Epidermal Wnt/beta-catenin signaling regulates adipocyte differentiation via secretion of adipogenic factors. Proc Natl Acad Sci USA 111(15):E1501–E1509
Driskell RR et al (2009) Sox2-positive dermal papilla cells specify hair follicle type in mammalian epidermis. Development 136(16):2815–2823
Dry FW (1926) The coat of the mouse (Mus musculus). J Genet 16(3):54
Elliott K, Stephenson TJ, Messenger AG (1999) Differences in hair follicle dermal papilla volume are due to extracellular matrix volume and cell number: implications for the control of hair follicle size and androgen responses. J Invest Dermatol 113(6):873–877

Enshell-Seijffers D et al (2010) Beta-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. Dev Cell 18(4):633–642
Fan SM et al (2018a) External light activates hair follicle stem cells through eyes via an ipRGC-SCN-sympathetic neural pathway. Proc Natl Acad Sci USA 115(29):E6880–E6889
Fan SM et al (2018b) Inducing hair follicle neogenesis with secreted proteins enriched in embryonic skin. Biomaterials 167:121–131
Festa E et al (2011) Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell 146(5):761–771
Foitzik K et al (2000) Control of murine hair follicle regression (catagen) by TGF-beta1 in vivo. FASEB J 14(5):752–760
Fujiwara H et al (2011) The basement membrane of hair follicle stem cells is a muscle cell niche. Cell 144(4):577–589
Furlan A et al (2016) Visceral motor neuron diversity delineates a cellular basis for nipple- and pilo-erection muscle control. Nat Neurosci 19(10):1331–1340
Fushimi T et al (2011) Narrow-band red LED light promotes mouse hair growth through paracrine growth factors from dermal papilla. J Dermatol Sci 64(3):246–248
Gilhar A, Paus R, Kalish RS (2007) Lymphocytes, neuropeptides, and genes involved in alopecia areata. J Clin Invest 117(8):2019–2027
Greco V et al (2009) A two-step mechanism for stem cell activation during hair regeneration. Cell Stem Cell 4(2):155–169
Guerrero-Juarez CF, Plikus MV (2018) Emerging nonmetabolic functions of skin fat. Nat Rev Endocrinol 14(3):163–173
Gur-Cohen S et al (2019) Stem cell-driven lymphatic remodeling coordinates tissue regeneration. Science 366:1218
Hansen LS et al (1984) The influence of the hair cycle on the thickness of mouse skin. Anat Rec 210(4):569–573
Harel S et al (2015) Pharmacologic inhibition of JAK-STAT signaling promotes hair growth. Sci Adv 1(9):e1500973
Headington JT (1987) Hair follicle biology and topical minoxidil: possible mechanisms of action. Dermatologica 175(Suppl 2):19–22
Heitman N et al (2020) Dermal sheath contraction powers stem cell niche relocation during hair cycle regression. Science 367(6474):161–166
Hibberts NA, Howell AE, Randall VA (1998) Balding hair follicle dermal papilla cells contain higher levels of androgen receptors than those from non-balding scalp. J Endocrinol 156(1):59–65
Ho WKW et al (2019) Feather arrays are patterned by interacting signalling and cell density waves. PLoS Biol 17(2):e3000132
Horne KA, Jahoda CA (1992) Restoration of hair growth by surgical implantation of follicular dermal sheath. Development 116(3):563–571
Hsu YC, Pasolli HA, Fuchs E (2011) Dynamics between stem cells, niche, and progeny in the hair follicle. Cell 144(1):92–105
Hsu YC, Li L, Fuchs E (2014a) Emerging interactions between skin stem cells and their niches. Nat Med 20(8):847–856
Hsu YC, Li L, Fuchs E (2014b) Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. Cell 157(4):935–949
Huang WY et al (2017a) Mobilizing transit-amplifying cell-derived ectopic progenitors prevents hair loss from chemotherapy or radiation therapy. Cancer Res 77(22):6083–6096
Huang WY et al (2017b) Stress-induced premature senescence of dermal papilla cells compromises hair follicle epithelial-mesenchymal interaction. J Dermatol Sci 86(2):114–122
Huang WY et al (2019) Anagen hair follicle repair: timely regenerative attempts from plastic extra-bulge epithelial cells. Exp Dermatol 28(4):406–412
Huang YC et al (2013) Scalable production of controllable dermal papilla spheroids on PVA surfaces and the effects of spheroid size on hair follicle regeneration. Biomaterials 34(2):442–451
Inaba M, Harn HI, Chuong CM (2019) Turing patterning with and without a global wave. PLoS Biol 17(3):e3000195

Inui S et al (2003) Identification of androgen-inducible TGF-beta1 derived from dermal papilla cells as a key mediator in androgenetic alopecia. J Investig Dermatol Symp Proc 8(1):69–71
Ito M et al (2002) Label-retaining cells in the bulge region are directed to cell death after plucking, followed by healing from the surviving hair germ. J Invest Dermatol 119(6):1310–1316
Ito M et al (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nat Med 11(12):1351–1354
Ito M et al (2007) Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. Nature 447(7142):316–320
Jahoda CA, Horne KA, Oliver RF (1984) Induction of hair growth by implantation of cultured dermal papilla cells. Nature 311(5986):560–562
Jaks V et al (2008) Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet 40(11):1291–1299
Jameson J, Havran WL (2007) Skin gammadelta T-cell functions in homeostasis and wound healing. Immunol Rev 215(1):114–122
Kaufman KD et al (1998) Finasteride in the treatment of men with androgenetic alopecia. Finasteride male pattern hair loss study group. J Am Acad Dermatol 39(4 Pt 1):578–589
Kollar EJ (1970) The induction of hair follicles by embryonic dermal papillae. J Invest Dermatol 55(6):374–378
Kreuzaler PA et al (2011) Stat3 controls lysosomal-mediated cell death in vivo. Nat Cell Biol 13(3):303–309
Kumamoto T et al (2003) Hair follicles serve as local reservoirs of skin mast cell precursors. Blood 102(5):1654–1660
Kwack MH et al (2008) Dihydrotestosterone-inducible dickkopf 1 from balding dermal papilla cells causes apoptosis in follicular keratinocytes. J Invest Dermatol 128(2):262–269
Kwack MH et al (2012) Dihydrotestosterone-inducible IL-6 inhibits elongation of human hair shafts by suppressing matrix cell proliferation and promotes regression of hair follicles in mice. J Invest Dermatol 132(1):43–49
Lai JJ et al (2012) The role of androgen and androgen receptor in skin-related disorders. Arch Dermatol Res 304(7):499–510
Lee P et al (2017) Stimulation of hair follicle stem cell proliferation through an IL-1 dependent activation of γδT-cells. Elife 6:366
Legue E, Nicolas JF (2005) Hair follicle renewal: organization of stem cells in the matrix and the role of stereotyped lineages and behaviors. Development 132(18):4143–4154
Legue E, Sequeira I, Nicolas JF (2010) Hair follicle renewal: authentic morphogenesis that depends on a complex progression of stem cell lineages. Development 137(4):569–577
Lei M et al (2014) Modulating hair follicle size with Wnt10b/DKK1 during hair regeneration. Exp Dermatol 23(6):407–413
Lei M, Yang L, Chuong CM (2017) Getting to the core of the dermal papilla. J Invest Dermatol 137(11):2250–2253
Lei MX, Chuong CM, Widelitz RB (2013) Tuning Wnt signals for more or fewer hairs. J Invest Dermatol 133(1):7–9
Leishman E et al (2013) Foxp1 maintains hair follicle stem cell quiescence through regulation of Fgf18. Development 140(18):3809–3818
Li KN et al (2019) Skin vasculature and hair follicle cross-talking associated with stem cell activation and tissue homeostasis. Elife 8
Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science 327(5965):542–545
Lin KK et al (2009) Circadian clock genes contribute to the regulation of hair follicle cycling. PLoS Genet 5(7):e1000573
Lindner G et al (1997) Analysis of apoptosis during hair follicle regression (catagen). Am J Pathol 151(6):1601–1617
Longest head hair (female) (2004) http://www.guinnessworldrecords.com/world-records/longest-hair-(female)

Lu CP et al (2016) Spatiotemporal antagonism in mesenchymal-epithelial signaling in sweat versus hair fate decision. Science 354(6319)
Maini PK, Baker RE, Chuong CM (2006) Developmental biology. The Turing model comes of molecular age. Science 314(5804):1397–1398
Mai-Yi Fan S et al (2018) Efficacy and safety of a low-level light therapy for androgenetic alopecia: a 24-week, randomized, double-blind, self-comparison, *sham device-controlled trial*. Dermatol Surg 44(11):1411–1420
Marshall WA, Tanner JM (1969) Variations in pattern of pubertal changes in girls. Arch Dis Child 44(235):291–303
Marshall WA, Tanner JM (1970) Variations in the pattern of pubertal changes in boys. Arch Dis Child 45(239):13–23
Matsumura H et al (2016) Hair follicle aging is driven by transepidermal elimination of stem cells via COL17A1 proteolysis. Science 351(6273):aad4395
Maurer M, Paus R, Czarnetzki BM (1995) Mast cells as modulators of hair follicle cycling. Exp Dermatol 4(4 Pt 2):266–271
Maurer M et al (1997) The mast cell products histamine and serotonin stimulate and TNF-alpha inhibits the proliferation of murine epidermal keratinocytes in situ. J Dermatol Sci 16(1):79–84
McConnell JD et al (1992) Finasteride, an inhibitor of 5 alpha-reductase, suppresses prostatic dihydrotestosterone in men with benign prostatic hyperplasia. J Clin Endocrinol Metab 74(3):505–508
McElwee KJ et al (2003) Cultured peribulbar dermal sheath cells can induce hair follicle development and contribute to the dermal sheath and dermal papilla. J Invest Dermatol 121(6):1267–1275
McGarvey EL et al (2001) Psychological sequelae and alopecia among women with cancer. Cancer Pract 9(6):283–289
Mendez-Ferrer S et al (2008) Haematopoietic stem cell release is regulated by circadian oscillations. Nature 452(7186):442–447
Mesa KR et al (2015) Niche-induced cell death and epithelial phagocytosis regulate hair follicle stem cell pool. Nature 522(7554):94–97
Millar SE (2002) Molecular mechanisms regulating hair follicle development. J Invest Dermatol 118(2):216–225
Mok KW et al (2019) Dermal condensate niche fate specification occurs prior to formation and is placode progenitor dependent. Dev Cell 48(1):32–48. e5
Muller-Rover S et al (2001) A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J Invest Dermatol 117(1):3–15
Murphy G et al (2001) The molecular determinants of sunburn cell formation. Exp Dermatol 10(3):155–160
Murray PJ et al (2012) Modelling hair follicle growth dynamics as an excitable medium. PLoS Comput Biol 8(12):e1002804
Myung PS et al (2013) Epithelial Wnt ligand secretion is required for adult hair follicle growth and regeneration. J Invest Dermatol 133(1):31–41
Nicu C et al (2019) Do human dermal adipocytes switch from lipogenesis in anagen to lipophagy and lipolysis during catagen in the human hair cycle? Exp Dermatol 28(4):432–435
Nyholt DR et al (2003) Genetic basis of male pattern baldness. J Invest Dermatol 121(6):1561–1564
Ohyama M et al (2006) Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest 116(1):249–260
Olsen EA et al (2006) The importance of dual 5alpha-reductase inhibition in the treatment of male pattern hair loss: results of a randomized placebo-controlled study of dutasteride versus finasteride. J Am Acad Dermatol 55(6):1014–1023
Orentreich N (1959) Autografts in alopecias and other selected dermatological conditions. Ann N Y Acad Sci 83:463–479
Osaka N et al (2007) ASK1-dependent recruitment and activation of macrophages induce hair growth in skin wounds. J Cell Biol 176(7):903–909

Oshima H et al (2001) Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104(2):233–245
Oshimori N, Fuchs E (2012) Paracrine TGF-beta signaling counterbalances BMP-mediated repression in hair follicle stem cell activation. Cell Stem Cell 10(1):63–75
Paladini RD et al (2005) Modulation of hair growth with small molecule agonists of the hedgehog signaling pathway. J Invest Dermatol 125(4):638–646
Paus R, Cotsarelis G (1999) The biology of hair follicles. N Engl J Med 341(7):491–497
Paus R et al (1994a) Mast cell involvement in murine hair growth. Dev Biol 163(1):230–240
Paus R et al (1994b) Chemotherapy-induced alopecia in mice. Induction by cyclophosphamide, inhibition by cyclosporine A, and modulation by dexamethasone. Am J Pathol 144(4):719–734
Paus R et al (1997) Transforming growth factor-beta receptor type I and type II expression during murine hair follicle development and cycling. J Invest Dermatol 109(4):518–526
Paus R et al (1998) Generation and cyclic remodeling of the hair follicle immune system in mice. J Invest Dermatol 111(1):7–18
Paus R, Muller-Rover S, Botchkarev VA (1999) Chronobiology of the hair follicle: hunting the "hair cycle clock". J Investig Dermatol Symp Proc 4(3):338–345
Paus R et al (2003) The hair follicle and immune privilege. J Investig Dermatol Symp Proc 8(2):188–194
Paus R et al (2013) Pathobiology of chemotherapy-induced hair loss. Lancet Oncol 14(2):e50–e59
Pena-Jimenez D et al (2019) Lymphatic vessels interact dynamically with the hair follicle stem cell niche during skin regeneration in vivo. EMBO J 38(19):e101688
Petukhova L et al (2010) Genome-wide association study in alopecia areata implicates both innate and adaptive immunity. Nature 466(7302):113–117
Philpott M, Paus R (1998) *Principles of hair follicle morphogenesis*. In: Chuong CM (ed) *Molecular basis of epithelial appendage morphogenesis*. R.G. Landers Company, Austin, pp 75–110
Plikus MV (2012) New activators and inhibitors in the hair cycle clock: targeting stem cells' state of competence. J Invest Dermatol 132(5):1321–1324
Plikus MV et al (2008) Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature 451(7176):340–344
Plikus MV et al (2011) Self-organizing and stochastic behaviors during the regeneration of hair stem cells. Science 332(6029):586–589
Plikus MV et al (2013) Local circadian clock gates cell cycle progression of transient amplifying cells during regenerative hair cycling. Proc Natl Acad Sci USA 110(23):E2106–E2115
Porter RM et al (2004) Functional analysis of keratin components in the mouse hair follicle inner root sheath. Br J Dermatol 150(2):195–204
Potten CS, Booth C, Pritchard DM (1997) The intestinal epithelial stem cell: the mucosal governor. Int J Exp Pathol 78(4):219–243
Rahmani W et al (2014) Hair follicle dermal stem cells regenerate the dermal sheath, repopulate the dermal papilla, and modulate hair type. Dev Cell 31(5):543–558
Rahmani W et al (2018) Macrophages promote wound-induced hair follicle regeneration in a CX3CR1- and TGF-beta1-dependent manner. J Invest Dermatol 138(10):2111–2122
Randall VA, Hibberts NA, Hamada K (1996) A comparison of the culture and growth of dermal papilla cells from hair follicles from non-balding and balding (androgenetic alopecia) scalp. Br J Dermatol 134(3):437–444
Randall VA et al (2000) The hair follicle: a paradoxical androgen target organ. Horm Res 54(5–6): 243–250
Reynolds AJ, Jahoda CA (1992) Cultured dermal papilla cells induce follicle formation and hair growth by trans differentiation of an adult epidermis. Development 115(2):587–593
Rishikaysh P et al (2014) Signaling involved in hair follicle morphogenesis and development. Int J Mol Sci 15(1):1647–1670
Rodeheffer MS, Birsoy K, Friedman JM (2008) Identification of white adipocyte progenitor cells in vivo. Cell 135(2):240–249

Rompolas P et al (2012) Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. Nature 487(7408):496–499
Rossi A et al (2012) Minoxidil use in dermatology, side effects and recent patents. Recent Patents Inflamm Allergy Drug Discov 6(2):130–136
Schneider MR, Schmidt-Ullrich R, Paus R (2009) The hair follicle as a dynamic miniorgan. Curr Biol 19(3):R132–R142
Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4(1–2):7–25
Sennett R, Rendl M (2012) Mesenchymal-epithelial interactions during hair follicle morphogenesis and cycling. Semin Cell Dev Biol 23(8):917–927
Sequeira I, Nicolas JF (2012) Redefining the structure of the hair follicle by 3D clonal analysis. Development 139(20):3741–3751
Sheen YS et al (2015) Visible red light enhances physiological anagen entry in vivo and has direct and indirect stimulative effects in vitro. Lasers Surg Med 47(1):50–59
Sick S et al (2006) WNT and DKK determine hair follicle spacing through a reaction-diffusion mechanism. Science 314(5804):1447–1450
Silver AF, Chase HB (1970) DNA synthesis in the adult hair germ during dormancy (telogen) and activation (early anagen). Dev Biol 21(3):440–451
Stenn KS, Paus R (2001) Controls of hair follicle cycling. Physiol Rev 81(1):449–494
Stöhr P (1903) Entwicklungsgeschichte des menschlichen Wollhaares. Anat Hefte 23:65
Strazzulla LC et al (2018) Alopecia areata: disease characteristics, clinical evaluation, and new perspectives on pathogenesis. J Am Acad Dermatol 78(1):1–12
Tang D et al (2019) The molecular machinery of regulated cell death. Cell Res 29(5):347–364
Thornton MJ et al (1993) Differences in testosterone metabolism by beard and scalp hair follicle dermal papilla cells. Clin Endocrinol 39(6):633–639
Tian H et al (2011) A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature 478(7368):255–259
Tobin DJ et al (2003) Plasticity and cytokinetic dynamics of the hair follicle mesenchyme during the hair growth cycle: implications for growth control and hair follicle transformations. J Investig Dermatol Symp Proc 8(1):80–86
Tumbar T et al (2004) Defining the epithelial stem cell niche in skin. Science 303(5656):359–363
Turksen K et al (1992) Interleukin 6: insights to its function in skin by overexpression in transgenic mice. Proc Natl Acad Sci USA 89(11):5068–5072
Unna P (1876) Beiträge zur Histologie und Entwiekelungsgeschichte der menschlichen Oberhaut und ihrer Anhangsgebilde. Arch Mikr Anat 12:29
Vanscott EJ, Ekel TM, Auerbach R (1963) Determinants of rate and kinetics of cell division in scalp hair. J Invest Dermatol 41:269–273
Wang E, McElwee KJ (2011) Etiopathogenesis of alopecia areata: why do our patients get it? Dermatol Ther 24(3):337–347
Wang ECE et al (2019) A subset of TREM2(+) dermal macrophages secretes Oncostatin M to maintain hair follicle stem cell quiescence and inhibit hair growth. Cell Stem Cell 24(4):654–669. e6
Wang EHC et al (2018) JAK inhibitors for treatment of alopecia Areata. J Invest Dermatol 138(9):1911–1916
Wang Q et al (2017a) A multi-scale model for hair follicles reveals heterogeneous domains driving rapid spatiotemporal hair growth patterning. Elife 6
Wang WH et al (2017c) Activation of mTORC1 signaling is required for timely hair follicle regeneration from radiation injury. Radiat Res 188(6):681–689
Wang X et al (2017b) Macrophages induce AKT/beta-catenin-dependent Lgr5(+) stem cell activation and hair follicle regeneration through TNF. Nat Commun 8(1)
Wilson A et al (2008) Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135(6):1118–1129
Wu YF et al (2015) Enhancing hair follicle regeneration by nonablative fractional laser: assessment of irradiation parameters and tissue response. Lasers Surg Med 47(4):331–341

Xie G et al (2015) Testing chemotherapeutic agents in the feather follicle identifies a selective blockade of cell proliferation and a key role for sonic hedgehog signaling in chemotherapy-induced tissue damage. J Invest Dermatol 135(3):690–700
Xin T et al (2018) Flexible fate determination ensures robust differentiation in the hair follicle. Nat Cell Biol 20(12):1361–1369
Xing L et al (2014) Alopecia areata is driven by cytotoxic T lymphocytes and is reversed by JAK inhibition. Nat Med 20(9):1043–1049
Yang H et al (2017) Epithelial-mesenchymal micro-niches govern stem cell lineage choices. Cell 169(3):483–496. e13
Young TH et al (2008) Self-assembly of dermal papilla cells into inductive spheroidal microtissues on poly(ethylene-co-vinyl alcohol) membranes for hair follicle regeneration. Biomaterials 29(26):3521–3530
Yu Z et al (2018) Hoxc-dependent mesenchymal niche heterogeneity drives regional hair follicle regeneration. Cell Stem Cell 23(4):487–500. e6
Zappacosta AR (1980) Reversal of baldness in patient receiving minoxidil for hypertension. N Engl J Med 303(25):1480–1481
Zhang B et al (2016a) Hair follicles' transit-amplifying cells govern concurrent dermal adipocyte production through sonic hedgehog. Genes Dev 30(20):2325–2338
Zhang B et al (2016b) Hair follicles' transit-amplifying cells govern concurrent dermal adipocyte production through Sonic Hedgehog. Genes Develop 30(20):2325–2338
Zhang LJ et al (2015) Innate immunity. Dermal adipocytes protect against invasive Staphylococcus aureus skin infection. Science 347(6217):67–71
Zhang YV et al (2009) Distinct self-renewal and differentiation phases in the niche of infrequently dividing hair follicle stem cells. Cell Stem Cell 5(3):267–278
Zhang Z et al (2019) Dermal adipose tissue has high plasticity and undergoes reversible dedifferentiation in mice. J Clin Invest 129(12):5327–*5342*
Zwick RK et al (2018) Anatomical, physiological, and functional diversity of adipose tissue. Cell Metab 27(1):68–83

Muse Cells

Mari Dezawa

Contents

Abstract

Multilineage-differentiating stress-enduring (Muse) cells were first reported in 2010. Since then, multiple groups have elucidated their unique properties. Muse cells are non-tumorigenic endogenous pluripotent-like stem cells that express pluripotency genes; are able to differentiate into triploblastic cells from a single cell and to self-renew; can be isolated as cells positive for a pluripotent stem cell surface marker, stage-specific embryonic antigen-3, from the connective tissue of various organs, as well as the bone marrow and peripheral blood; preferentially

M. Dezawa (✉)
Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, Sendai, Japan
e-mail: mdezawa@med.tohoku.ac.jp

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_63

migrate to damaged tissue after systemic administration; and spontaneously differentiate into tissue-compatible cells after homing, which enables them to deliver structural and functional recovery with few safety concerns. These properties of Muse cells enable therapeutic effects in only a few simple steps, namely, collection from easily accessible tissue sources, expansion, and administration by intravenous injection. Because Muse cells are naturally existing stem cells with unique reparative functions, they may furnish a novel therapeutic concept compatible with the body's natural repair system, "reparative medicine," that does not rely on introducing or manipulating artificial genes.

1 Introduction

Multilineage-differentiating stress-enduring (Muse) cells were first reported in 2010 as non-tumorigenic endogenous stem cells with pluripotent-like properties that reside in the bone marrow (BM) as well as in cultured BM-mesenchymal stem cells (MSCs) and dermal fibroblasts (Kuroda et al. 2010). Muse cells express pluripotency genes, although the expression levels are lower than those in embryonic stem (ES) cells and induced pluripotent stem (iPS) cells but are higher than those in somatic cells, and are able to differentiate into endodermal-, mesodermal-, and ectodermal-lineage cells from a single cell. This triploblastic differentiation ability is self-renewable, demonstrating the pluripotent-like characteristics of these unique cells (Kuroda et al. 2010; Wakao et al. 2011; Kuroda et al. 2013). Based on studies by several groups, Muse cells exhibit unique properties not demonstrated by other pluripotent stem cells; for example, Muse cells reside not only in the BM but also in the connective tissue of various organs, and they are stress-tolerant (Wakao et al. 2011; Kinoshita et al. 2015; Mineda et al. 2015; Alessio et al. 2017). They also circulate in the peripheral blood in healthy donors, and the number of Muse cells increases in response to severe damage in the body, such as stroke (Hori et al. 2016). Remarkably, topically or intravenously administered naïve Muse cells preferentially home to the damaged site and spontaneously differentiate into tissue-compatible cells after integration, where they deliver tissue reparative effects. This series of actions is observed in animal model tissue damage, such as skin ulcer (Kinoshita et al. 2015), stroke (Yamauchi et al. 2015; Uchida et al. 2016, 2017), chronic kidney disease (Uchida et al. 2017), and liver injury and cirrhosis (Katagiri et al. 2015; Iseki et al. 2017), and Muse cells replace damaged cells spanning from ectodermal to mesodermal and endodermal lineages due to their pluripotent-like properties.

Observations of both endogenous and exogenous Muse cells suggest the fundamental importance of Muse cells; they are assumed to participate in daily, minute reparative body maintenance. Whereas Muse cells exhibit triploblastic differentiation and self-renewability, similar to ES and iPS cells, the fundamental difference between Muse cells and ES/iPS cells is that Muse cells are *naturally existing* endogenous stem cells that appear to have an ongoing role in the body. In the following sections, we provide an overview of the unique properties of Muse cells

and their reparative effects in several models of tissue damage and discuss future perspectives for the clinical application of Muse cells.

2 General Features of Muse Cells

Muse cells are isolated as cells positive for the pluripotent stem cell surface marker, stage-specific embryonic antigen (SSEA)-3, from various sources including the BM, peripheral blood, and the connective tissue of various organs (Kuroda et al. 2010, 2013; Wakao et al. 2011; Heneidi et al. 2013; Ogura et al. 2014; Gimeno et al. 2017) (Fig. 1). They express pluripotency markers and exhibit the ability to generate cells representative of all three germ layers from a single cell and to self-renew in vitro, while their telomerase activity is at the somatic cell level and they do not form teratomas when transplanted in vivo, consistent with the fact that they normally reside in adult tissues (Kuroda et al. 2010; Wakao et al. 2011; Ogura et al. 2014; Gimeno et al. 2017).

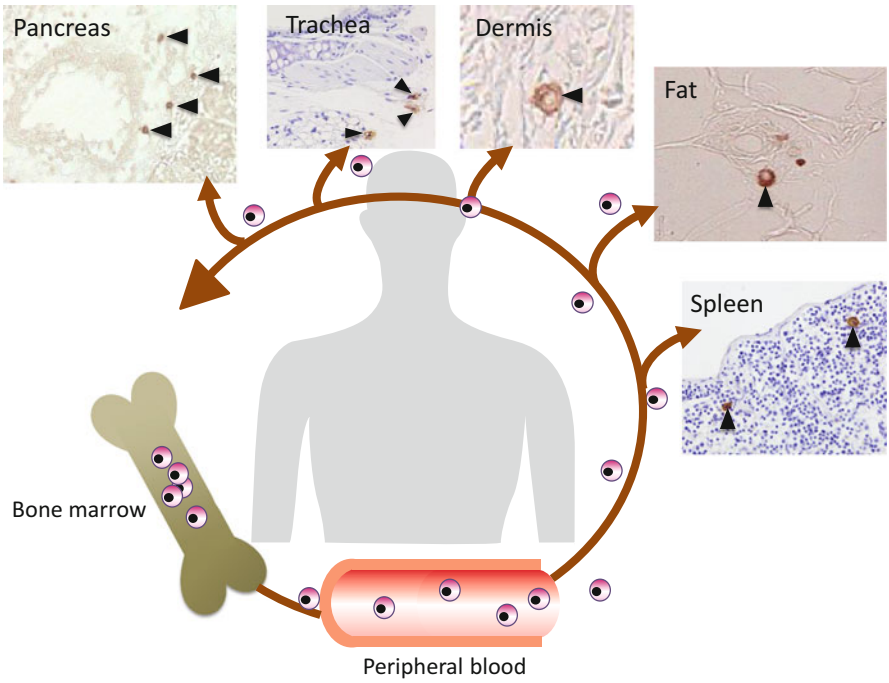


Fig. 1 Distribution of Muse cells in vivo. Muse cells, labeled with SSEA-3, are located in the bone marrow, peripheral blood, and the connective tissue of various organs (black arrowheads) (Dezawa 2016; Hori et al. 2016). Therefore, bone marrow-derived Muse cells are assumed to be mobilized into the peripheral blood and then distributed to organs via the connective tissue

2.1 Muse Cells as Stress-Tolerant Stem Cells

Muse cells were initially identified as stress-tolerant stem cells (Kuroda et al. 2010). When human BM-MSCs and dermal fibroblasts were treated with long-term trypsin (LTT) incubation over 16 h without any nutrients, a subpopulation of these cultured cells that was largely positive for SSEA-3(+) survived, while the remainder of the cells died due to severe stress. This is similar to other tissue stem cells in that they are stress-tolerant and, while normally dormant, they are activated by stimuli such as stress and enter into the cell cycle (Hong et al. 2009; Qiu et al. 2009).

Recently, Alessio et al. (2017) performed a secretome analysis and demonstrated that, compared to BM-MSCs and adipose-derived stem cells (ADSCs), Muse cells produce a greater amount of factors related to stress tolerance and suppression of apoptosis. Muse cells appear to secrete factors that may preserve their stem cell features, allowing them to survive under stress conditions and contributing to their immunomodulatory capacity. For example, the 14-3-3 proteins (also named YWHAQ, YWHAG, YWHAE, YWHAZ, and YWHAB) are a family of highly conserved acidic 30-kDa molecules that form stable homo- and heterodimers and bind to their phosphoserine- and phosphothreonine-containing ligands to regulate a wide range of cellular phenomena (Gardino and Yaffe 2011; Clapp et al. 2012). Several studies found that 14-3-3 proteins play a key role in regulating the cell cycle and the cell response to DNA damage following internal or external injury and act like chaperonin to reduce cellular stress and subsequent apoptosis. Furthermore, some 14-3-3 isoforms can inactivate the pro-apoptotic protein BAD by preventing its negative effects on the pro-survival protein Bcl-XL (Gardino and Yaffe 2011; Clapp et al. 2012). The fact that Muse cell secretomes contain most of the 14-3-3 isoforms involved in anti-apoptotic activity strongly supports their stress-enduring capacity (Alessio et al. 2017).

Some upstream regulatory cytokines, such as corticotropin-releasing factor and leukemia inhibitory factor, found in Muse cells but not in MSCs and ADSCs, may have a role in controlling stem cell identity, self-renewal, and protection from pro-apoptotic stress in Muse cells. Bone morphogenetic protein 4 and fibroblast growth factor 18, growth factors identified as Muse cell-specific upstream regulators, are also not produced by MSCs and ADSCs. Muse cells are involved in heightening stem cell differentiation potential and controlling cell commitment (Alessio et al. 2017).

These results suggest that Muse secretomes have components that act in an autocrine/paracrine manner to promote cell survival and preserve stemness.

2.2 Tissue Distribution of Muse Cells

Muse cells were first identified from the human BM aspirate and the dermis (Kuroda et al. 2010). Subsequently, they were also identified from adipose tissue by stress selection (Heneidi et al. 2013). Human Muse cells, directly collected from BM aspirate, express pluripotency markers, differentiate into triploblastic lineages, and

reside in the BM where they form cluster-like structures (Hori et al. 2016). In addition to these tissues, Muse cells are located sparsely in the connective tissue of various organs, including the trachea, spleen, and umbilical cord, while they are not observed in particular histologic structures (Dezawa 2016) (Fig. 1). This is in contrast to MSCs, which are located close to the subendothelial regions of small blood vessels in the connective tissue (Galderisi and Giordano 2014), and other tissue stem cells, such as neural and hematopoietic stem cells, whose niches were described previously (Reinhard et al. 2016; Beerman et al. 2017). Hori et al. (2016) recently demonstrated that Muse cells are also located in the peripheral blood of healthy donors at a very low ratio (3–4 cells/μl), and the number sharply increases 24 h after the onset of stroke. Based on these findings together, Muse cells are thought to reside in the BM, gradually mobilize to the peripheral blood, and then distribute to organ connective tissues (Fig. 1).

2.3 Pluripotent-Like Properties

Muse cells not only express the pluripotent surface marker SSEA-3, but they express other pluripotent markers as well, such as octamer binding transcription factor 3/4 (Oct3/4), sex-determining region Y box 2 (Sox2), Nanog, and reduced expression 1 (Rex1) (Kuroda et al. 2010, 2013; Wakao et al. 2011; Heneidi et al. 2013; Ogura et al. 2014). The expression *pattern* of the pluripotency-related genes is similar between Muse and ES/iPS cells, whereas the expression *level* is lower in Muse cells compared to that in ES/iPS cells. Importantly, non-Muse MSCs, i.e., cells other than Muse cells among MSCs, do not express pluripotency genes, in sharp contrast to Muse cells (Wakao et al. 2011). Consistent with this observation, promoter regions of Oct3/4 and Nanog are less methylated in Muse cells than in non-Muse MSCs (Wakao et al. 2011).

Proteins belonging to the (i) protein kinase A signaling, (ii) FXR/RXR activation, and (iii) LXR/RXR activation pathways are highly expressed in Muse cells compared with MSCs and ADSCs, suggesting roles for these factors in the stemness of Muse cells (Alessio et al. 2017). The protein kinase A pathway is reported to play a role in self-renewal and differentiation of mouse ES cells (Faherty et al. 2007), and the FXR/RXR and LXR/RXR activation pathways promote self-renewal and maintenance of the multipotency of several stem cell types (Ho et al. 2013). Other protein factors, such as POU2F1, DLX4, BCL11A, KLF5, GATA3, AIRE, and FLH2, are also expressed in Muse cells and are suggested to be involved in governing stem cell functions such as self-renewal, cell fate specification, and cell survival under stress (Alessio et al. 2017).

The pluripotency of Muse cells is remarkably higher in suspension than in an adherent state. In adherent culture, Muse cells have a morphology typical of mesenchymal cells such as fibroblasts, but after incubation in suspension for 6–8 h, the morphology drastically changes and becomes similar to that of the embryoid bodies of ES cells formed in suspension (Kuroda et al. 2010; Wakao et al. 2011; Kuroda et al. 2013) (Fig. 2). Not only the morphology of Muse cells changes in suspension,

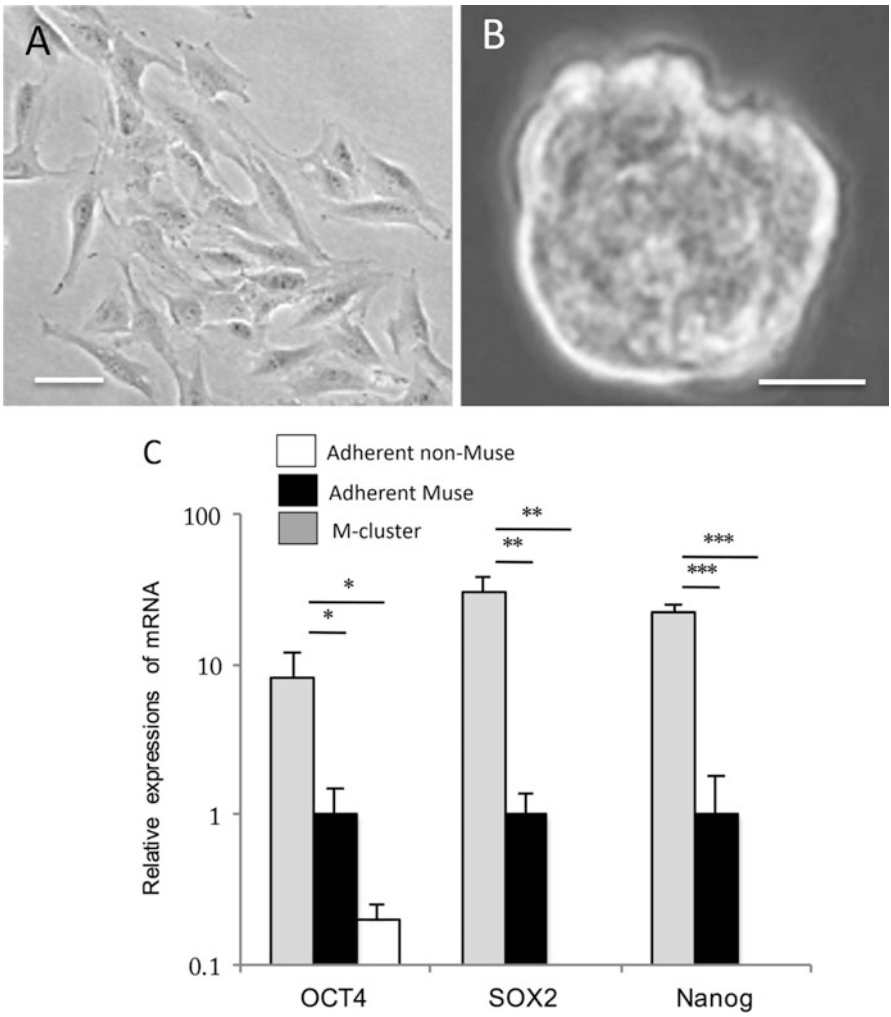


Fig. 2 Pluripotency of Muse cells in adherent and suspension conditions. (**a**) Muse cells were isolated as SSEA-3(+) by fluorescence-activated cell sorting and cultured in adherent conditions. (**b**) Picture reproduced from Dezawa (2016). Single Muse cell-derived clusters (M-cluster) formed in suspension have a morphology similar to that of ES cell-derived embryoid bodies formed in suspension. Bars: 50 μm. (**c**) Data reproduced from Iseki et al. (2017). Quantitative polymerase chain reaction for octamer-binding transcription factor 4 (OCT4), sex-determining region Y-box 2 (SOX2), and Nanog in adherent non-Muse MSCs, adherent Muse cells, and M-clusters formed from single cells in suspension. $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$

but also the gene expression levels of Oct3/4, Nanog, and Sox2 are 10–80 times higher in suspension (Iseki et al. 2017) (Fig. 2). How pluripotent gene expression is controlled in suspension and adherent culture systems is an interesting topic for future studies.

2.4 Differentiation In Vitro and In Vivo

Similar to ES cells, Muse cells spontaneously differentiate into cells representative of all three germ layers on gelatin-coated culture plates (Kuroda et al. 2010). Muse cells in suspension culture begin to divide and form cell clusters (M-clusters) that appear similar to ES cell-derived embryoid bodies by day 7–14. The M-clusters spontaneously generate cells positive for neurofilament (ectodermal), smooth muscle actin (mesodermal), and alpha-fetoprotein (endodermal) without cytokine induction or gene introduction when transferred to gelatin-coated adherent culture dishes and allowed to expand (Kuroda et al. 2010; Ogura et al. 2014) (Fig. 3). While the proportion of differentiation marker-positive cells is only several percent for endodermal and ectodermal lineages and 10% for mesodermal lineage, this demonstrates the ability of the Muse cells to generate cells representative of all three germ layers from a single cell (Kuroda et al. 2010).

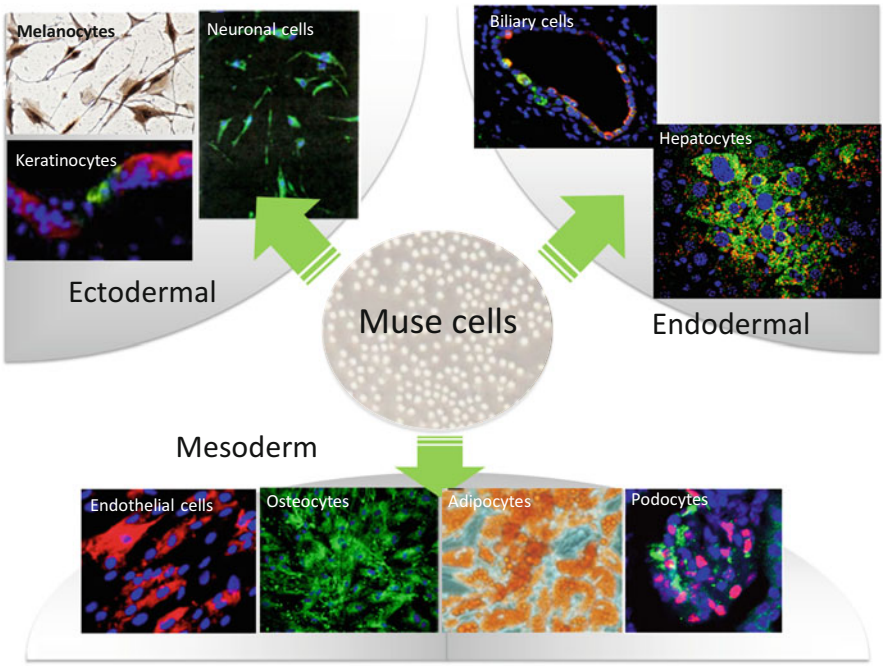


Fig. 3 Triploblastic differentiation ability of Muse cells. Muse cells are able to differentiate in vitro (either by spontaneous differentiation on a gelatin-coated culture dish or by cytokine induction) and in vivo (administration to damage models) into ectodermal-, endodermal-, and mesodermal-lineage cells. Differentiation into melanocytes (L-DOPA reaction) (Tsuchiyama et al. 2013), neuronal cells (MAP-2) (Wakao et al. 2011), endothelial cells (smooth muscle actin) (Kuroda et al. 2010), osteocytes (osteocalcin), adipocytes (oil red) (Ogura et al. 2014) was observed in vitro. Differentiation of all cell types, except endothelial cells, was induced by cytokine application. Keratinocytes (Kuroda et al. 2010), hepatocytes (Iseki et al. 2017), biliary cells (Katagiri et al. 2015), and podocytes (Uchida et al. 2017) were obtained from tissue damage models

Not only do Muse cells spontaneously differentiate, they also differentiate at a high rate (80–95%) into fatty acid-binding protein 4-positive adipocytes and albumin-positive hepatocyte- and neurofilament-positive neural-lineage cells under cytokine induction (Ogura et al. 2014) (Fig. 3). They also differentiate into functional melanocytes when the appropriate cytokines are supplied (Tsuchiyama et al. 2013). A set of 10 factors (including wingless-type mouse mammary tumor virus integration site family, member 3A, endothelin-3, linoleic acid, stem cell factor, and dexamethasone) newly evoke expression of melanocyte markers, tyrosinase-related protein 1, glycoprotein 100, dopachrome tautomerase, and tyrosinase, and Muse cells become pigment-producing functional melanocytes that are positive for the l-3,4-dihydroxyphenylalanine (L-DOPA) reaction. In fact, these Muse cell-derived melanocytes produce melanin pigments that transfer to neighboring cells when transplanted in vivo, suggesting melanocyte functionality (Tsuchiyama et al. 2013).

2.5 Self-Renewability of Muse Cells

Muse cells exhibit self-renewability, which is a characteristic of pluripotency (Ogura et al. 2014). Single Muse cell-derived M-clusters generated in suspension culture were collected, and half of the clusters were transferred individually onto gelatin culture to allow the cells to expand out of the M-clusters, and then their expression of triploblastic lineage markers, microtubule-associated protein 2 (MAP-2), guanine-adenine-thymine-adenine-binding protein 6 (GATA6), alpha-fetoprotein, and NK2 homeobox 5 was confirmed (Ogura et al. 2014) (Fig. 4). The remainder of the clusters were individually transferred to adherent culture and allowed to proliferate, after which they underwent a second round of single-cell suspension culture to generate second-generation M-clusters. This experimental cycle was repeated, and M-clusters from each step exhibited triploblastic differentiation at the single-cell level (Fig. 4). These findings support the pluripotent-like property of Muse cells.

2.6 Difference Between Muse and Non-muse MSCs

When MSCs are separated into Muse cells and the remaining cells, non-Muse MSCs, these two populations contrast sharply in several ways (Table 1). First, while both populations express general MSC surface markers, such as CD29, CD90, and CD105, Muse cells also express pluripotent SSEA-3. Second, pluripotency gene expression in non-Muse MSCs is little to nonexistent compared with that in Muse cells (Wakao et al. 2011). Third, unlike Muse cells, non-Muse MSCs are unable to survive more than a couple of days and cannot proliferate from a single cell to form clusters in suspension (Kuroda et al. 2010; Wakao et al. 2011). Therefore, non-Muse MSCs do not demonstrate self-renewability. Most importantly, non-Muse MSCs sharply contrast with Muse cells in their differentiation potential. Non-Muse MSCs are unable to cross lineage boundaries between the original mesoderm to ectoderm or endoderm lineages. In fact, even in the presence of cytokine cocktails, non-Muse MSCs show only partial intracellular responses to cytokine induction, but they do not differentiate into hepatocytes

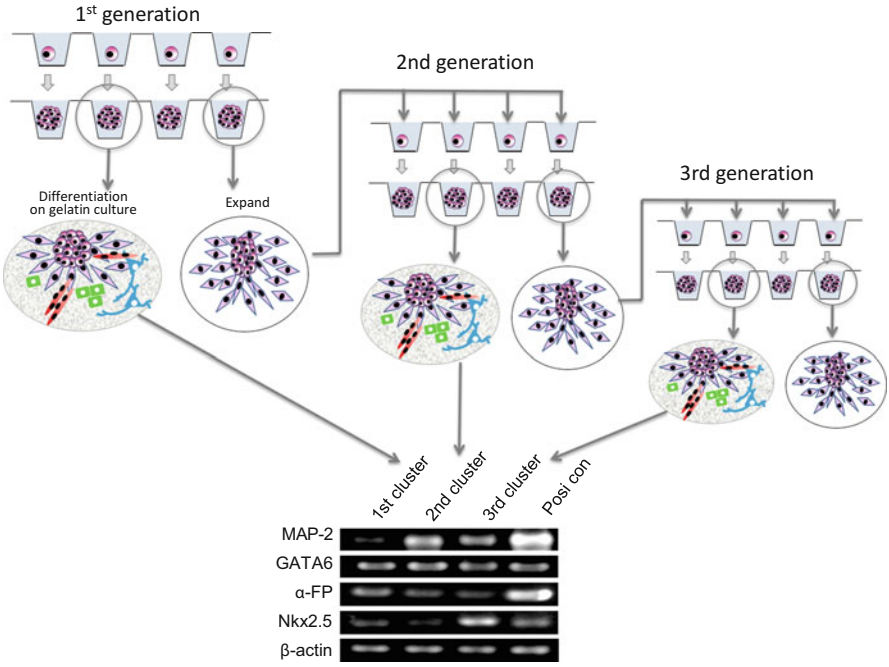


Fig. 4 Triploblastic differentiation ability is self-renewable in Muse cells. Single Muse cell-derived clusters formed in suspension show spontaneous triploblastic differentiation in a gelatin-coated culture dish. Other single cell-derived clusters are expanded in adherent culture, and then subjected to single cell suspension culture to produce 2nd generation differentiated cells. Single Muse cell-derived clusters show triploblastic differentiation. This cycle is repeated for a 3rd generation. Thus, the pluripotency-like properties of Muse cells are self-renewable (Ogura et al. 2014). (Pictures adapted with permission from Ogura et al. (2014))

(endodermal)- or neuronal (ectodermal)-lineage cells nor into melanocytes (ectodermal) (Wakao et al. 2011; Tsuchiyama et al. 2013; Ogura et al. 2014). For induction of melanocytes, even the addition of the ten factors mentioned above is not sufficient to convert non-Muse MSCs into functional melanocytes, and only a partial response, temporary expression of tyrosinase-related protein 1 is evoked, but other melanocyte markers such as dopachrome tautomerase, glycoprotein 100, and tyrosinase do not appear (Tsuchiyama et al. 2013). Such a difference between Muse and non-Muse MSCs is consistent with the fact that Muse cells express a subset of pluripotency genes that are not expressed in non-Muse MSCs.

In contrast to ectodermal- and endodermal-lineage cells, non-Muse MSCs are able to differentiate into osteocytes, cartilage cells, and adipocytes in the presence of cytokines, but their differentiation rate is lower, and the time required for their differentiation is longer than those for Muse cells (Ogura et al. 2014). For example, fatty acid-binding protein 4-positive adipocytes are generated by Muse cells at an efficiency higher than 72% within 1 week, but non-Muse MSCs produce them with only ~30% efficiency within 3 weeks.

Table 1 Comparison of muse cells and non-muse MSCs

	Muse cells	Non-muse MSCs
Surface marker	SSEA-3, CD105, CD90, CD29	CD105, CD90, CD29,
Pluripotent marker	Oct3/4, Sox2, Nanog, Rex1	Negative or very low
Endodermal differentiation	Hepatocytes, biliary cells,	(−), partial response to induction in vitro
Ectodermal differentiation	Neuronal cells, oligodendrocytes, melanocytes, keratinocytes	(−) partial response to induction in vitro
Mesodermal differentiation	Glomerular cells, skeletal muscle, smooth muscle,	(−)
Osteocytes	(+)	(+) lower efficiency
Adipocytes	(+)	(+) lower efficiency
Migration to damaged tissue	(+)	(−)
Survival in damaged tissue	(+)	(−)
Tumorigenicity	(−)	(−)

The ability of non-Muse MSCs to survive and integrate into damaged tissue in vivo is also different from that of Muse cells. While Muse cells remain in damaged tissues for at least 2–3 months as differentiated cells in models of stroke and kidney and liver damages, non-Muse MSCs basically do not remain in the damaged tissue for a short-term period such as 1–2 weeks after administration (Katagiri et al. 2015; Yamauchi et al. 2015; Uchida et al. 2016, 2017; Iseki et al. 2017). In an acute liver damage model, Muse and non-Muse MSCs are both mainly located in the lung and spleen several days after intravenous administration, but 2 weeks later, the majority of the Muse cells are located in the damaged liver and to a lesser extent in the lung, whereas non-Muse MSCs are located only in the lung and were undetectable in the damaged liver (Iseki et al. 2017) (Fig. 5). This same tendency was observed in a stroke model following topical infusion (Yamauchi et al. 2015; Uchida et al. 2016, 2017).

Because non-Muse MSCs comprise ~99% of MSCs, the actions of non-Muse MSCs can be expected to account for the majority of the representative activities of MSCs. Considering the difference between Muse and non-Muse MSCs, Muse cells are expected to produce better results in tissue survival, cell replacement, and structural and functional regeneration than MSCs. The differences between Muse and non-Muse MSCs are summarized in Table 1.

3 Beneficial Characteristics of Muse Cells for Reparative Medicine

3.1 Non-tumorigenicity

Because Muse cells reside naturally in our bodies, they are non-tumorigenic. Their gene expression levels of tumorigenic factors and telomerase activity are equivalent to those in somatic cells and are substantially lower than those in ES/iPS cells

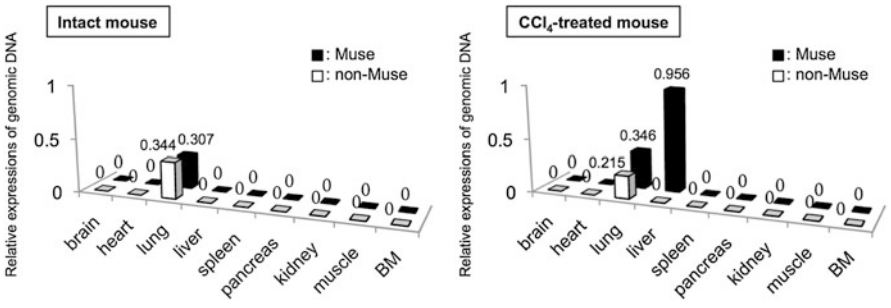


Fig. 5 Preferential homing of intravenously injected Muse cells to damaged tissue. In vivo dynamics of human Muse and non-Muse MSCs in a SCID mouse-liver damage model induced by intraperitoneal injection of carbon tetrachloride (CCL_4). Cells were administered 24 h after CCl4 injection. Quantitative polymerase chain reaction of a human-specific Alu sequence both in intact (**a**) and CCl_4-treated mice (**b**) injected with human Muse and non-Muse MSCs at 2 weeks (*BM* bone marrow). (Data are reproduced with permission from Iseki et al. (2017))

(Wakao et al. 2011). When transplanted into Nog mouse testes, human Muse cells from both the BM and adipose tissue do not generate teratomas, unlike undifferentiated ES and iPS cells, for up to 6 months (Kuroda et al. 2010; Ogura et al. 2014; Gimeno et al. 2017).

The proportion of Muse cells in the BM-mononucleated fraction is ~0.03%, namely, 1 of 3000 cells (Kuroda et al. 2010). This indicates that Muse cells have been included in BM transplantations into leukemia patients for many years with no tumorigenicity, indirectly supporting their safety with respect to not being tumorigenic in humans.

Migration toward damaged tissue: One of the prominent features of Muse cells is that they migrate to and home into damaged tissue following systemic administration. The migratory activity of human Muse cells was examined using the serum from a mouse model of chronic kidney disease (CKD) induced by intravenous administration of doxorubicin (Adriamycin) (Uchida et al. 2017). Muse cells exhibited strong in vitro migratory activity toward the serum, while human non-Muse MSCs did not move from the baseline level, even in the presence of the CKD serum.

A similar tendency was observed in a liver damage model. Muse cells showed a significantly higher level of migration toward the serum and liver tissue 24 h after liver damage than non-Muse MSCs.

The in vivo dynamics of intravenously injected (tail vein) human Muse and non-Muse MSCs were analyzed in both CKD and acute liver damage models (Iseki et al. 2017; Uchida et al. 2017). After several weeks, Muse cells exhibited a high level of homing to the kidney in the CKD model and to the liver in the acute liver damage model, while non-Muse MSCs are not detected in those damaged organs (Iseki et al. 2017; Uchida et al. 2017) (Fig. 5).

Together, these results indicate that Muse cells have a much higher ability to migrate to and accumulate in the damaged tissue both in vitro and in vivo, while non-Muse MSCs do not exhibit such ability.

Several factors are reported to control the migration of MSCs. The ligands for stem cell-derived factor-1 (CXCR4) and hepatocyte growth factor (c-Met) play important roles in the homing of MSCs to the damaged liver (Son et al. 2006; Ma et al. 2014). In Western blotting experiments, CXCR4 and c-Met signals were expressed to a similar extent between human Muse and non-Muse MSCs, and AMD3100, a CXCR4 antagonist, partially suppressed the migration of both Muse and non-Muse MSCs toward the serum from the liver damage model (Iseki et al. 2017). Therefore, the CXCR4 stem cell-derived factor-1 axis is considered to participate in Muse cell migration, while the factors that explain the different migration activity between Muse and non-Muse MSCs remain to be clarified.

3.2 Differentiation into Tissue-Compatible Cells After Integration

In addition to triploblastic differentiation in vitro, Muse cells also exhibit their unique differentiation ability in vivo. Following administration and homing to the damaged site, they differentiate into tissue-compatible cells. In a stroke model, Muse cells spontaneously differentiated into MAP-2(+)/NeuN(+) neural cells that were able to reconstruct pyramidal and sensory tracts with pyramidal decussation and synaptic connection with host neurons (Uchida et al. 2016, 2017). In the liver cirrhosis model, they were shown to differentiate into hepatocyte specific antigen 1 (HepPar-1)-positive/albumin-positive/anti-trypsin-positive hepatocytes that also expressed enzymes relevant to liver function (Iseki et al. 2017). These differentiations were achieved spontaneously because the administered Muse cells were naïve cells that were untreated. The pluripotent-like properties of Muse cells do not explain the tissue-compatible differentiation. Recently, Iseki et al. (2017), based on studies of damaged hepatocytes, suggested that differentiation of Muse cells is mainly controlled by direct interactions and/or contact between Muse cells and damaged cells in vitro, and not by humoral factors produced by the host cells. Host cells that instruct Muse cells must be damaged, and the direct interaction or contact between Muse cells and intact host cells does not evoke Muse cell differentiation.

3.3 Suppression of Fibrosis by Muse Cells

Apart from cell replacement, Muse cells have another beneficial effect, suppression of fibrosis. This effect was described in liver cirrhosis and CKD models (Iseki et al. 2017; Uchida et al. 2017). Muse cells have the ability to produce several different matrix metalloproteases, namely, metalloproteases-1, metalloproteases-2, and metalloproteases-9, which are involved in fibrolysis and/or suppression of fibrosis (Duarte et al. 2015). Because Muse cells integrate and remain in the damaged tissue for a longer period while non-Muse MSCs do not, suppression of fibrosis was statistically higher in Muse cell-administered groups compared with vehicle and non-Muse MSC groups in liver cirrhosis and CDK models (Iseki et al. 2017; Uchida et al. 2017).

4 Reparative Effects of Human Muse Cells in Animal Models

As mentioned above, systemically administered Muse cells preferentially migrate and home to damaged tissue and differentiate into tissue-compatible cells after integration (Fig. 6). This series of events enables Muse cells to replenish lost cells in damaged tissue, leading to structural and functional regeneration. In this section, we describe the spontaneous differentiation of systemically and topically administered human Muse cells into endodermal (hepatocytes), ectodermal (neuronal cells), and mesodermal (glomerular cells) cells after migration and integration into models of liver damage, stroke, and CKD.

4.1 Liver Damage Models

A study using a partial hepatectomy model showed the ability of Muse cells to differentiate not only into hepatocytes but also into cells of other liver components, the biliary duct, sinusoid endothelial cells, and Kupffer cells, after integrating into the damaged liver. Human BM-Muse cells were intravenously

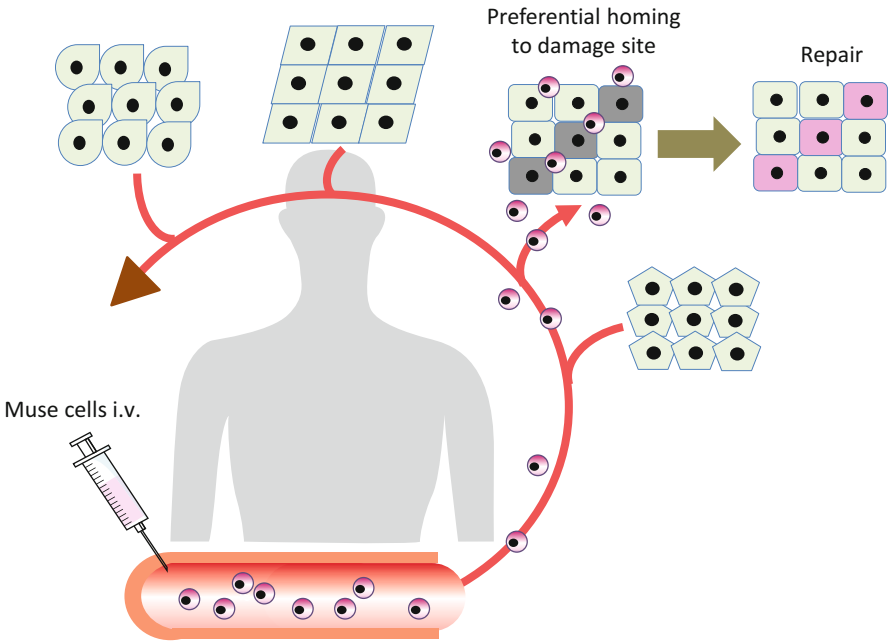


Fig. 6 Reparative effects of Muse cells after intravenous injection. Muse cells preferentially migrate to and accumulate into the damaged tissue after intravenous injection. They repair the tissue by spontaneously differentiating into tissue-compatible cells to replenish lost or damaged cells

injected into severe combined immune deficiency (SCID) mice with partial hepatectomy (Katagiri et al. 2015). Homing of Muse cells was observed 2 days after the administration, mostly at the transected border, and they expressed human cytokeratin 19, delta-like leucine zipper kinase, oval cell marker 6, and alpha-fetoprotein, markers of human liver progenitors. At 2 weeks, Muse cells began to express human-specific HepPar-1, albumin, and α1-antitrypsin, markers of hepatocytes, as well as cytokeratin 7 and lymphatic vessel endothelial hyaluronan receptor 1, markers of cholangiocytes and sinusoidal endothelial cells, respectively, whereas a very small number of cells positive for liver progenitor markers were still detected. At 4 weeks, in addition to the above mature markers, Muse cells that expressed CD68 (Kupffer cells) were detected, and 13.1% of hepatocytes (HepPar-1 cells) in the transection border were considered to derive from Muse cells. In contrast to Muse cells, non-Muse MSCs were not detected from 1 week to over the entire period, suggesting that non-Muse MSCs did not home to the liver in the early phase (Katagiri et al. 2015).

In the SCID mouse liver cirrhosis model, human BM-Muse cells exhibit high migration and homing capacities to the liver, but not to other organs except, to a lesser extent, the lungs at 2 weeks after intravenous injection (Iseki et al. 2017). After homing, Muse cells spontaneously differentiated into HepPar-1- ($71.1 \pm 15.2\%$), human albumin- ($54.3 \pm 8.2\%$), and anti-trypsin ($47.9 \pm 4.6\%$)-positive cells without fusing with host hepatocytes and express mature functional markers, such as human-CYP1A2, an enzyme involved in drug metabolism, and human glucose 6-phosphatase, an enzyme related to free glucose formation, at 8 weeks. Serum total bilirubin and albumin concentrations and attenuation of fibrosis are significantly recovered in the Muse cell-transplanted group but not in the control groups, which received vehicle or the same number of non-Muse MSCs (Iseki et al. 2017).

4.2 Stroke Models

The structural and functional regenerative capacity of human Muse cells was evaluated in two models, an acute phase transient middle cerebral artery occlusion model (Uchida et al. 2016) and a subacute phase lacuna infarct model (Uchida et al. 2017). Human dermal Muse cells (30,000 cells) were stereotaxically injected as three deposits into the rat ischemic cortex 2 days after transient middle cerebral artery occlusion, and the mice were examined over 84 days (Uchida et al. 2016). Muse cells spontaneously committed to neural/neuronal-lineage cells; at day 3, they had already started to extend neurite-like processes and expressed NeuroD and Mash1, markers of neuronal progenitor cells. At day 7, they displayed extensions of neurite-like processes more abundantly compared to day 3, started to form elaborate connections and networks with each other, and expressed MAP-2, NeuN, and human doublecortin, markers of neuronal cells. Approximately 65% of Muse cells survived in the host cortex and differentiated into neuronal cells that expressed NeuN, MAP-2, and calbindin, and ~25% of Muse cells expressed the oligodendrocyte marker glutathione S-transferase-π at 84 days, but glial fibrillary acidic protein-positive astrocytes were scarcely observed. In contrast to Muse cells,

non-Muse MSCs were not detected at 84 days. While integration and differentiation into neural lineages were clearly observed in the cortex injected with Muse cells, statistically meaningful improvements in neurologic and motor functions became evident ~2.5 months after transplantation. Importantly, this suggested that the recovery was not due to a bystander effect of Muse cells but rather due to reconstruction of neuronal circuits through integration of Muse cells into the damaged site, differentiation into neuronal cells, extension of neuritis, and reconstruction of connections with the proper target neurons. Indeed, Muse cells that integrated into the sensory-motor cortex extended their neurites into the contralateral spinal cord, and synapses that formed between human Muse cells and host neurons in the sensory cortex resulted in the recovery of hind limb somatosensory evoked potentials (Uchida et al. 2016).

In the lacuna stroke model, 1×10^5 human BM-derived Muse cells were administered into the perilesion brain 2 weeks after lacunar infarction in SCID mice (Uchida et al. 2017). Approximately 28% of the initially transplanted Muse cells remained in the host brain at 8 weeks and differentiated into cells expressing NeuN (~62%), MAP2 (~30%), and glutathione S-transferase-π (~12%). Dextran tracing revealed the formation of new connections between host cortex neurons and Muse cells at the lesion site as well as between Muse cells and neurons at the anterior horn of the spinal cord. Notably, Muse cells extended neurites through the ipsilateral pyramidal tract, crossed to the contralateral side, and reached the pyramidal tract in the dorsal funiculus of the spinal cord, demonstrating the reconstruction of the pyramidal decussation. Those animals displayed significant recovery of locomotor function, as assessed using the cylinder test. The recovery of function was reversed by administering the human-selective diphtheria toxin, indicating that the functional recovery was due to the presence of Muse cells and not to bystander effects of Muse cells (Uchida et al. 2017).

Together, these results suggested that administration of Muse cells not only at the acute phase but also at the subacute phase replenishes lost neuronal cells by spontaneously differentiating into neural cells and oligodendrocytes and facilitating neural circuit reconstruction to produce statistically meaningful functional recovery.

4.3 Chronic Kidney Disease Model

Human BM-derived Muse cells were systemically administered into SCID and BALB/c mouse models of doxorubicin-induced focal segmental glomerulosclerosis without concurrent administration of immunosuppressants (Uchida et al. 2017). The human Muse cells preferentially integrated into the damaged glomeruli. In both SCID and BALB/c models, Muse cells spontaneously differentiated into podocyte (podocin; ~30%), mesangial cell (megsin; ~13%), and endothelial cell (CD31; 40–46%) marker-positive cells without fusing to the host cells; attenuated glomerular sclerosis and interstitial fibrosis; and induced the recovery of renal function. Notably, at 5 weeks the BALB/c model showed the most impressive improvement in urine protein, creatinine clearance, and plasma creatinine levels, despite xenotransplantation without concurrent immunosuppressant administration. In contrast to the

SCID model, functional improvement in the BALB/c model was impaired at 7 weeks, however, due to subsequent immunorejection, suggesting the importance of Muse cell survival as glomerular cells for tissue repair and functional recovery (Uchida et al. 2017).

5 Conclusions

Muse cells are unique stem cells that (1) are non-tumorigenic endogenous pluripotent stem cells; (2) preferentially migrate to damaged tissue; (3) exhibit stress tolerance, enabling them to survive the harsh microenvironment of damage tissue; (3) spontaneously differentiate into tissue-compatible endodermal-, ectodermal- and mesodermal-lineage cells after integration; and (4) deliver structural and functional recovery. These properties render unnecessary the induction of Muse cells into target cells prior to transplantation, unlike ES and iPS cells. Furthermore, these unique properties enable Muse cells to repair tissues in a few simple steps: namely, collection and expansion from easily accessible tissue sources such as the BM and adipose tissue and intravenous injection. Therefore, Muse cells, with their impressive regenerative performance, may provide a simple feasible strategy for treating a variety of diseases due to their ability to differentiate into triploblastic cells. Such a simple and accessible approach may lower the hurdle of regenerative medicine, allowing cell therapy to be applied to general medicine.

A critical issue regarding the use of stem cell/progenitors cells for tissue reconstruction is the low survival rate of the cells after administration. The harsh environment in the damaged tissue due to pro-apoptotic and pro-inflammatory factors and reactive oxygen and nitrogen species makes it very difficult for transplanted cells to survive in the damaged tissue (Song et al. 2009; Arumugam et al. 2010). Because such a hostile environment is difficult to control, a realistic solution is to utilize stress-tolerant cells, which could significantly improve the homing and survival rates. Muse cells fulfill such a need and are adaptable to the severe conditions that are present in damaged tissue (Alessio et al. 2017).

From the standpoint of clinical treatment, allogeneic cells are more practical than autologous cells because autologous cells require more time to collect and expand to obtain a sufficient number of Muse cells for clinical application and would therefore not be available to acute-phase patients or patients with basic diseases. The findings in the CKD-BALB/c model were impressive because, even without administration of immunosuppressants, human Muse cells survived in the immunologically normal BALB/c glomerulus as podocytes, mesangial cells, and endothelial cells and delivered improvement in functional parameters for up to 5 weeks, although they were eventually rejected at 7 weeks (Uchida et al. 2017). The immunomodulatory effects of Muse cells were demonstrated by their inhibition of T lymphocyte proliferation in a mixed lymphocyte proliferation assay and their expression of indoleamine 2,3 dioxygenase following stimulation with interferon-gamma (Uchida et al. 2017). Therefore, similar to MSCs, allogeneic cell therapy might be possible with Muse cells.

Because Muse cells are naturally existing stem cells with unique reparative functions, they may open the door to a "Next-Generation Medical Care" compatible with the body's natural repair system, namely, "reparative medicine," that does not rely on artificial gene introduction or manipulation.

References

Alessio N, Ozcan S, Tatsumi K, Murat A, Peluso G, Dezawa M, Galderisi U (2017) The secretome of MUSE cells contains factors that may play a role in regulation of stemness, apoptosis and immunomodulation. Cell Cycle 16(1):33–44

Arumugam TV, Phillips TM, Cheng A, Morrell CH, Mattson MP, Wan R (2010) Age and energy intake interact to modify cell stress pathways and stroke outcome. Ann Neurol 67(1):41–52

Beerman I, Luis TC, Singbrant S, Lo Celso C, Mendez-Ferrer S (2017) The evolving view of the hematopoietic stem cell niche. Exp Hematol. https://doi.org/10.1016/j.exphem.2017.01.008

Clapp C, Portt L, Khoury C, Sheibani S, Norman G, Ebner P, Eid R, Vali H, Mandato CA, Madeo F, Greenwood MT (2012) 14-3-3 protects against stress-induced apoptosis. Cell Death Dis 3:e348

Dezawa M (2016) Muse cells provide the pluripotency of mesenchymal stem cells: direct contribution of Muse cells to tissue regeneration. Cell Transplant 25(5):849–861

Duarte S, Baber J, Fujii T, Coito AJ (2015) Matrix metalloproteinases in liver injury, repair and fibrosis. Matrix Biol 44–46:147–156

Faherty S, Fitzgerald A, Keohan M, Quinlan LR (2007) Self-renewal and differentiation of mouse embryonic stem cells as measured by Oct4 expression: the role of the cAMP/PKA pathway. In Vitro Cell Dev Biol Anim 43(1):37–47

Galderisi U, Giordano A (2014) The gap between the physiological and therapeutic roles of mesenchymal stem cells. Med Res Rev 34(5):1100–1126

Gardino AK, Yaffe MB (2011) 14-3-3 proteins as signaling integration points for cell cycle control and apoptosis. Semin Cell Dev Biol 22(7):688–695

Gimeno ML, Fuertes F, Barcala Tabarrozzi AE, Attorressi AI, Cucchiani R, Corrales L, Oliveira TC, Sogayar MC, Labriola L, Dewey RA, Perone MJ (2017) Pluripotent nontumorigenic adipose tissue-derived Muse cells have immunomodulatory capacity mediated by transforming growth factor-beta1. Stem Cells Transl Med 6(1):161–173

Heneidi S, Simerman AA, Keller E, Singh P, Li X, Dumesic DA, Chazenbalk G (2013) Awakened by cellular stress: isolation and characterization of a novel population of pluripotent stem cells derived from human adipose tissue. PLoS One 8(6):e64752

Ho TC, Chen SL, Wu JY, Ho MY, Chen LJ, Hsieh JW, Cheng HC, Tsao YP (2013) PEDF promotes self-renewal of limbal stem cell and accelerates corneal epithelial wound healing. Stem Cells 31(9):1775–1784

Hong HS, Lee J, Lee E, Kwon YS, Lee E, Ahn W, Jiang MH, Kim JC, Son Y (2009) A new role of substance P as an injury-inducible messenger for mobilization of CD29(+) stromal-like cells. Nat Med 15(4):425–435

Hori E, Hayakawa Y, Hayashi T, Hori S, Okamoto S, Shibata T, Kubo M, Horie Y, Sasahara M, Kuroda S (2016) Mobilization of pluripotent multilineage-differentiating stress-enduring cells in ischemic stroke. J Stroke Cerebrovasc Dis 25(6):1473–1481

Iseki M, Kushida Y, Wakao S, Akimoto T, Mizuma M, Motoi F, Asada R, Shimizu S, Unno M, Chazenbalk G, Dezawa M (2017) Muse cells, nontumorigenic pluripotent-like stem cells, have liver regeneration capacity through specific homing and cell replacement in a mouse model of liver fibrosis. Cell Transplant 26(5):821–840

Katagiri H, Kushida Y, Nojima M, Kuroda Y, Wakao S, Ishida K, Endo F, Kume K, Takahara T, Nitta H, Tsuda H, Dezawa M, Nishizuka SS (2015) A distinct subpopulation of bone marrow mesenchymal stem cells, Muse cells, directly commit to the replacement of liver components. Am J Transplant. https://doi.org/10.1111/ajt.13537

Kinoshita K, Kuno S, Ishimine H, Aoi N, Mineda K, Kato H, Doi K, Kanayama K, Feng J, Mashiko T, Kurisaki A, Yoshimura K (2015) Therapeutic potential of adipose-derived SSEA-3-positive Muse cells for treating diabetic skin ulcers. Stem Cells Transl Med 4(2):146–155

Kuroda Y, Kitada M, Wakao S, Nishikawa K, Tanimura Y, Makinoshima H, Goda M, Akashi H, Inutsuka A, Niwa A, Shigemoto T, Nabeshima Y, Nakahata T, Fujiyoshi Y, Dezawa M (2010) Unique multipotent cells in adult human mesenchymal cell populations. Proc Natl Acad Sci U S A 107(19):8639–8643

Kuroda Y, Wakao S, Kitada M, Murakami T, Nojima M, Dezawa M (2013) Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. Nat Protoc 8(7):1391–1415

Ma HC, Shi XL, Ren HZ, Yuan XW, Ding YT (2014) Targeted migration of mesenchymal stem cells modified with CXCR4 to acute failing liver improves liver regeneration. World J Gastroenterol 20(40):14884–14894

Mineda K, Feng J, Ishimine H, Takada H, Doi K, Kuno S, Kinoshita K, Kanayama K, Kato H, Mashiko T, Hashimoto I, Nakanishi H, Kurisaki A, Yoshimura K (2015) Therapeutic potential of human adipose-derived stem/stromal cell microspheroids prepared by three-dimensional culture in non-cross-linked hyaluronic acid gel. Stem Cells Transl Med 4(12):1511–1522

Ogura F, Wakao S, Kuroda Y, Tsuchiyama K, Bagheri M, Heneidi S, Chazenbalk G, Aiba S, Dezawa M (2014) Human adipose tissue possesses a unique population of pluripotent stem cells with nontumorigenic and low telomerase activities: potential implications in regenerative medicine. Stem Cells Dev 23(7):717–728

Qiu J, Takagi Y, Harada J, Topalkara K, Wang Y, Sims JR, Zheng G, Huang P, Ling Y, Scadden DT, Moskowitz MA, Cheng T (2009) p27Kip1 constrains proliferation of neural progenitor cells in adult brain under homeostatic and ischemic conditions. Stem Cells 27(4):920–927

Reinhard J, Brosicke N, Theocharidis U, Faissner A (2016) The extracellular matrix niche microenvironment of neural and cancer stem cells in the brain. Int J Biochem Cell Biol 81(Pt A): 174–183

Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, Ratajczak MZ, Janowska-Wieczorek A (2006) Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells 24(5):1254–1264

Song J, Finnerty CC, Herndon DN, Boehning D, Jeschke MG (2009) Severe burn-induced endoplasmic reticulum stress and hepatic damage in mice. Mol Med 15(9–10):316–320

Tsuchiyama K, Wakao S, Kuroda Y, Ogura F, Nojima M, Sawaya N, Yamasaki K, Aiba S, Dezawa M (2013) Functional melanocytes are readily reprogrammable from multilineage-differentiating stress-enduring (Muse) cells, distinct stem cells in human fibroblasts. J Invest Dermatol 133(10):2425–2435

Uchida H, Morita T, Niizuma K, Kushida Y, Kuroda Y, Wakao S, Sakata H, Matsuzaka Y, Mushiake H, Tominaga T, Borlongan CV, Dezawa M (2016) Transplantation of unique subpopulation of fibroblasts, Muse cells, ameliorates experimental stroke possibly via robust neuronal differentiation. Stem Cells 34(1):160–173

Uchida H, Niizuma K, Kushida Y, Wakao S, Tominaga T, Borlongan CV, Dezawa M (2017) Human Muse cells reconstruct neuronal circuitry in subacute lacunar stroke model. Stroke 48(2):428–435

Uchida N, Kushida Y, Kitada M, Wakao S, Kumagai N, Kuroda Y, Kondo Y, Hirohara Y, Kure H, Chazenbalk G, Dezawa M (2017) Beneficial effects of systemically administered human Muse cells in murine adriamycin nephropathy. J Am Soc Nephrol 28(10):2946–2960

Wakao S, Kitada M, Kuroda Y, Shigemoto T, Matsuse D, Akashi H, Tanimura Y, Tsuchiyama K, Kikuchi T, Goda M, Nakahata T, Fujiyoshi Y, Dezawa M (2011) Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. Proc Natl Acad Sci U S A 108(24):9875–9880

Yamauchi T, Kuroda Y, Morita T, Shichinohe H, Houkin K, Dezawa M, Kuroda S (2015) Therapeutic effects of human multilineage-differentiating stress enduring (MUSE) cell transplantation into infarct brain of mice. PLoS One 10(3):e0116009

Embryonic Stem Cells

Philip Lewis, Edina Silajdžić, Daniel R. Brison, and Susan J. Kimber

Contents

Philip Lewis and Edina Silajdžić contributed equally to this work.

P. Lewis (✉) · E. Silajdžić · D. R. Brison · S. J. Kimber
Faculty of Biology Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK
e-mail: philip.lewis-6@manchester.ac.uk; edina.silajdzic@manchester.ac.uk; daniel.brison@manchester.ac.uk; sue.kimber@manchester.ac.uk

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_19

Abstract

Embryonic stem cells (ESCs) are pluripotent stem cells derived from a preimplantation embryo. ESCs are distinguished by two major properties: their pluripotency (the ability to differentiate into all derivatives of the three primary germ layers) and their ability to replicate indefinitely under defined conditions. Human ESCs (hESCs) can be used to study early human development and genetic disease and for in vitro toxicology testing. Because of their plasticity and potentially unlimited capacity for self-renewal, clinical-grade hESC therapies have been proposed for tissue replacement after injury or disease. In this chapter we summarize the process of hESC derivation, discuss characterization (the standard tests that are performed during the cell culture process to check that the cells exhibit the fundamental properties that make them ESCs), and provide examples of protocols that are used to induce hESCs to differentiate into specific cell types.

1 Introduction

Embryonic stem cells (ESCs) are pluripotent stem cells derived from preimplantation embryos. Several features define ESCs, but the two key properties that make these cells remarkable are their capacity for indefinite self-renewal and their ability to give rise to all cell types derived from the three embryonic germ layers. The characteristics and culture methods used for ESCs vary greatly between species; as such, this review will focus exclusively on human ESCs (hESCs), although we will reference discoveries in other species such as mouse for historical context and development of the field. In addition, induced pluripotent stem cell (iPSC) lines will be referred to throughout for comparative purposes. Human ESCs and iPSCs will be referred to collectively as human pluripotent stem cells (hPSCs).

2 Origin and Derivation of hESCs

hESCs are typically derived from the inner cell mass (ICM) of blastocyst stage embryos on days 5–8 after fertilization. The blastocyst is composed of two distinct cell types: the ICM, which later becomes the epiblast and goes on to give rise to the fetus, and the trophectoderm (TE) (Fig. 1a). The principal role of the TE in early development is to regulate the microenvironment in which the ICM develops. As development continues the TE will form the extraembryonic support structures required for successful development of the embryo, such as the placenta and extraembryonic membranes.

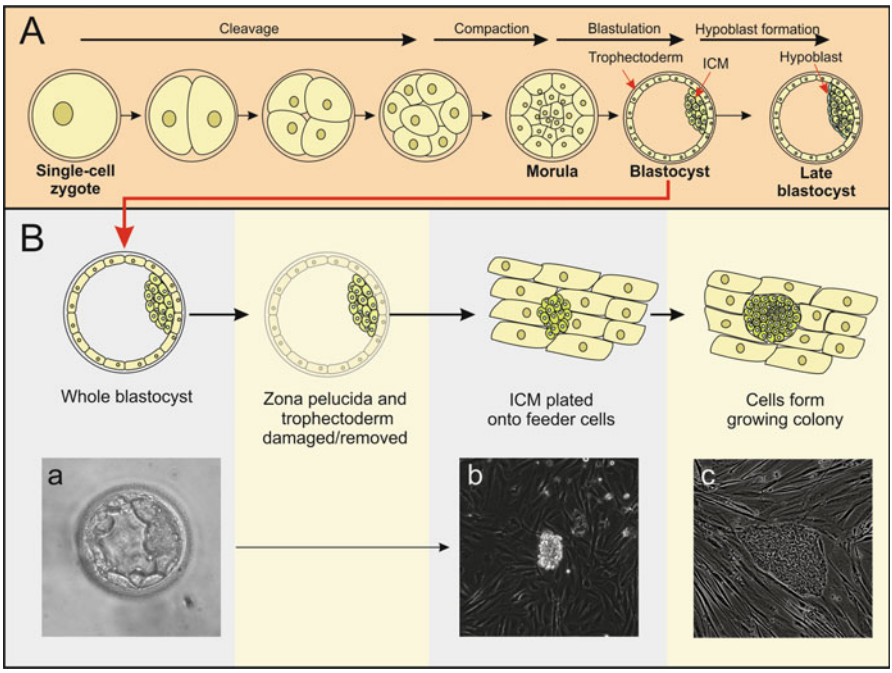


Fig. 1 Origin and derivation of hESCs. (**a**) Early human embryonic development, progressing from single-cell zygote to late blastocyst. Cells for hESC derivation are taken from early blastocyst stage embryos, typically prior to formation of the hypoblast, as indicated. (**b**) Derivation of hESCs from the blastocyst. Cells are derived from the whole blastocyst as shown in the phase contrast image (**a**); the zona pellucida is then commonly removed enzymatically before the isolated ICM is separated from the TE and plated onto feeder cells (**b**). After some days, these ICM cells begin to form a hESC colony among the feeder cells (**c**)

The ICM is formed from the inner cells of the morula, while the TE forms from the outer cells. During blastocyst formation the ICM becomes localized to one side of the TE vesicle, adjacent to the forming fluid-filled blastocyst cavity. The ICM is later separated from the blastocyst cavity by the formation of a second extraembryonic layer of cells, the hypoblast, becoming the early epiblast (Fig. 1a). While the cells of the TE begin to form a specialized support structure, the ICM cells remain undifferentiated, fully pluripotent and proliferative and progress to form all of the tissues and structures of the human body. It is from the pluripotent cells of the ICM that hESC cells are derived.

2.1 Derivation

ESCs are typically derived using a variety of techniques from microsurgery to antibody or even laser-assisted methodologies (Solter and Knowles 1975; Thomson et al. 1998; Reubinoff et al. 2000; Strom et al. 2007; Turetsky et al. 2008). Although

there are differences in these methods, the aim of each is the same: to remove and separate the ICM from the TE so that the cells of the ICM can be cultured and expanded in vitro (Fig. 1b). Derivation without separation of the ICM and TE has been accomplished; however, the presence of highly proliferative TE cells can suppress growth of ESC cells and their co-culture is typically avoided (Heins et al. 2004; Inzunza et al. 2005). All of the above methodologies require the destruction of the blastocyst, which has raised concerns among some religious groups. Alternative methodologies have been developed which generate hESC lines from stages other than blastocyst, including as early as the four-cell embryo (Geens et al. 2009), and indeed using only single blastomeres from the morula, a technique which leaves a potentially viable embryo (Chung et al. 2008; Klimanskaya et al. 2006, 2007), While use of blastomeres biopsied from the embryo in this way to generate hESC lines might overcome one objection to the use of human embryos, arguably the potential damage incurred to the embryo raises another equally valid objection. A number of other efforts have been made to mitigate any concerns surrounding stem cell derivation, by utilizing eggs or embryos which would otherwise be discarded from the IVF process. These include deriving cells from oocytes unsuitable for IVF following parthenogenetic activation (De Sousa et al. 2009; Camarasa et al. 2012), from growth-arrested IVF embryos (Zhang et al. 2006), and from otherwise nonviable or poor-quality embryos (Gavrilov et al. 2011; Lerou et al. 2008; Ye et al. 2017).

Conventionally, the separated, expanded ICM-derived cells are then cultured in medium which promotes pluripotent stem cell growth, on a substrate formed of a lawn of non-mitotic "feeder" cells, such as mitotically inactivated mouse embryonic fibroblasts (MEFs) (Thomson et al. 1998; Crocco et al. 2013). Islands of cells emerge with the classical ESC morphology of small cells with a high nucleus to cytoplasm ratio and prominent nucleoli. If derivation is successful, then the ESCs will form a growing colony of pluripotent stem cells within the lawn of feeder cells (Fig. 1b). As this colony grows care must be taken to only expand pluripotent stem cells, as the cells are prone to spontaneous differentiation, particularly at the periphery of the colony (Rosowski et al. 2015). Once the colony reaches a threshold size, the cells can be passaged manually by physical dissection of the colony into smaller pieces, which allows the separation of morphologically ESC-like cells from those showing evidence of differentiation. Passaged cells are moved to a fresh plate of feeder cells where they form fresh colonies, and expansion continues this way until the stability of the ESC line has been established, usually defined as the ability to cryopreserve and successfully resuscitate the line (Masters and Stacey 2007; Murdoch et al. 2012).

3 Culture of hESCs

In general the conditions and methods of hESC culture are similar to those for other mammalian cells; cells are cultured in plates or flasks; in an isotonic, nutrient-rich medium; and in a humid 37 °C incubator at typically 5% CO_2 in air. However, hESC

culture differs from many standard mammalian cell culture systems in three key attributes which are essential to maintaining pluripotency: media, substrate, and passaging method.

3.1 Media and Substrates

3.1.1 Substrates

The principal point of variance in the way hESCs can be grown is that of feeder-dependent or feeder-free culture. hESCs require a combination of signaling molecules, both soluble and adherent, to maintain their pluripotency and growth. Initially the provision of the, then unknown, signaling factors was facilitated by growing colonies of hESCs on a lawn of non-mitotic MEFs.

The dependence on the use of MEFs was problematic as they are of nonhuman origin, and the composition of signaling and extracellular proteins that they secrete and present can be highly variable between batches and labs (Lim and Bodnar 2002; Chin et al. 2007; Crocco et al. 2013). Despite many advances in substrates and media, there is not yet a robust, universally accepted feeder-free protocol for the derivation and culture of karyotypically normal hESC lines (International Stem Cell Initiative et al. 2011). In order to avoid the potential contamination of these cultures with animal-derived pathogens, a wide variety of xeno-free feeder cells have been developed such as human dermal, foreskin, placental, or embryonic fibroblasts (HDFs, HFFs, HPFs, or HEFs) (Crocco et al. 2013; Mckay et al. 2011). These cell lines have been demonstrated to reliably replace MEFs, removing our reliance on them when deriving hESC lines.

Once hESC lines have been stably derived onto feeder cells, they can be moved to feeder-free conditions, in which tissue culture plastic is pre-coated with proteins or protein mixtures prior to the addition of cells. Matrigel, an extracellular matrix (ECM) protein preparation derived from Engelbreth–Holm–Swarm mouse sarcoma cells, has been used for over 30 years to culture ECM-dependent cells (Kleinman et al. 1982; Bissell et al. 1987) and for over 15 years to culture hESCs in feeder-free conditions (Xu et al. 2001). While Matrigel has allowed hESCs to be cultured feeder-free, it is still an animal-derived, undefined product, containing a variable mixture of laminins, collagen IV, proteoglycans, and signaling molecules. To avoid this variability, researchers looked into use of cell-binding proteins, or protein motifs, which could be recombinantly produced and be immobilized onto tissue culture plastic, such as vitronectin (Braam et al. 2008) or laminins (Rodin et al. 2010). These proteins enable initial cell attachment allowing cells to then produce their own extracellular matrix permitting pluripotent growth of cells (Soteriou et al. 2013) with activation of focal adhesion kinase (Vitillo et al. 2016). The recombinant N-terminal vitronectin domain (VTN-N) is a particularly noteworthy substrate due to the cost-efficiency of its production (Braam et al. 2008). VTN-N is now used routinely with media such as E8 (see below) to culture pluripotent stem cells in completely xeno-free, defined conditions (Chen et al. 2011).

3.1.2 Media

As with all cell lines, hESCs require a complex mixture of nutrients and growth factors for successful in vitro culture. This medium generally needs to be changed daily. Initial success in culturing hESCs in vitro was achieved in medium containing serum (a complex, undefined mixture of nutrients and signaling factors). While these conditions allow the robust expansion and culture of pluripotent hESCs, with the use of MEFs and Matrigel, as discussed above (Sect. 3.1.1), there is a batch-to-batch variation in the content of serum, and the use of animal-derived products adds additional technical and safety considerations for applications in regenerative medicine. As such there has been a consistent drive toward synthesizing a fully xeno-free and defined medium.

The progress away from undefined animal-derived sera has been rapid. Initially the culture-staple fetal calf serum (FCS) was improved upon by utilizing partly defined or synthetic serum replacements such as knockout serum replacement (KOSR), synthetic serum substitute (SSS), or StemPro (Weathersbee et al. 1995; Lee et al. 2006; Swistowski et al. 2009). Fully defined medium such as TeSR reduced and defined the number of components in hESC medium to a supplement of 18 components added to a DMEM/F12 basal media of 52 components (Chen et al. 2011; Ludwig et al. 2006b). This formula was later improved in TeSR2 by making all components xeno-free in origin (Meng et al. 2012).

TeSR medium was formulated methodically by removing individual components from stem cell culture medium and determining whether their removal had any negative impact on cell pluripotency (Ludwig et al. 2006b). This reductive methodology was improved through a combinatorial approach taking into account component interactions, and it was revealed that it was possible to remove further factors without negatively affecting the cells. This resulted in the simplest medium to date containing only seven components added to a DMEM/F12 basal media, Essential 8 (Chen et al. 2011). These components are DMEM/F12 medium, L-ascorbic acid, selenium, transferrin, $NaHCO_3$, insulin, FGF2, and TGFβ1.

3.1.3 Passaging Methods

In understanding the behavior of hESCs in culture, it is informative to consider that a growing hESC culture is poised to continue the developmental progress of the ICM and begin differentiation. The pluripotent stem cell population is present only transiently in the embryo; for about 24–48 h before being lost to cell commitment. In order to maintain the pluripotency of a hESC culture, the cells are prevented from becoming over confluent. Cells are typically allowed to proliferate for only 2–3 days, at which point they are "passaged" disaggregating them into small clumps which will form new colonies. All passaging methods work by dissociating the cells from the culture surface and/or each other and then seeding a portion of these cells into a fresh culture vessel. hESC cultures are passaged either as clumps or as single cells and consequently will either grow in colonies or in a continuous culture monolayer (Fig. 2).

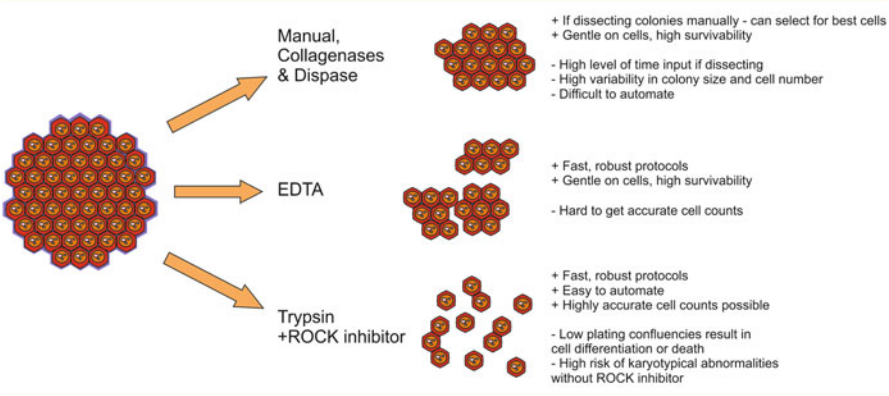


Fig. 2 Common methods of hESC passage (Based on Beers et al. 2012). Once a hESC culture reaches a threshold density, it is disaggregated into smaller clusters of cells, or to single cells, a portion of which are then plated onto a new culture surface; this process is called cell passaging. Each passaging method has advantages (+) and disadvantages (−), some of which are listed above

Manual Passage

The presence of cell–cell contacts between hESCs in culture helps to maintain their pluripotency (Studeny et al. 2002). Therefore, hESCs are typically passaged and cultured in clumps and colonies, rather than being individualized by dissociation to single cells as is common with other mammalian cells (including mouse ESCs). Manual passage of hESCs is often used in early stages of cell line derivation and is particularly advantageous as it can allow for the enrichment of healthy, pluripotent stem cells in a colony of mixed quality. Cells can be passaged manually either by dissection, which can very precisely enrich for specific groups of cells, or by use of a cell scraper which is a less labor-intensive method employed when selection of certain cells is not necessary.

Enzymatic Dissociation

Enzymatic methods of dissociation utilize enzymes such as trypsin to digest the extracellular adhesion domains and proteins which bind cells to each other and/or to their substrate. The use of enzymes which target exclusively the extracellular matrix produced by cells provides a gentler dissociation method which maintains cells in large clumps and colonies; however, their use is often restricted to particular substrates. Collagenases, for example, can be used to gently dissociate colonies cultured on feeder cells, whereas dispases are more effective in feeder-free culture.

Trypsin

While the above methods allow for healthy passage of hESCs, the operator variability in the size of colonies generated and the number of cells passaged make it difficult to accurately quantify cell number when cells are passaged as clumps. Enzymatic passage using trypsin avoids this and is a robust, reproducible, and easily automated method of hESC passage. Additionally, GMP-grade recombinant trypsin is available

and widely used in the field, which is advantageous for translating cultures to regenerative therapies (Ellerstrom et al. 2007). Trypsin breaks down the extracellular connections both between cells and between cells and their substrate. However, culturing hESCs in this manner has been reported to decrease viability and pluripotency in some studies (Brimble et al. 2004), and the surviving culture may become enriched for cells with karyotypic abnormalities (Sect. 4.4). The loss of viability and pluripotency by single-cell passage can be avoided by supplementing media with an inhibitor of the protein Rho-associated protein kinase (ROCK) for 24 h following passage (Watanabe et al. 2007). It has been suggested that ROCK inhibition disrupts extracellular cues that would normally induce detachment-induced apoptosis (anoikis) when the cells are dissociated and enhances cell–cell interaction through modulation of cadherins and GAP junctions, leading to the formation of small aggregates of hESCs in suspension (Krawetz et al. 2009; Wang et al. 2009). The predominant mechanism appears to be thorough myosin light chain phosphorylation (Chen et al. 2010) and so inhibition of actin–myosin contraction. There are persistent concerns in the field that long-term culture in the presence of ROCK inhibitor may carry the risk of an increased incidence of karyotypic abnormalities, although a definitive study demonstrating this has yet to appear.

Ethylenediaminetetraacetic Acid (EDTA)
Rather than digesting extracellular proteins, EDTA treatment functions by chelating calcium, which is required for cell–cell adhesion and integrin binding. This results in a less harsh passage, with retention of clusters of up to tens of cells and minimal damage to cell surface proteins. The larger clusters of cells resulting from EDTA passage remove the necessity of ROCK inhibitor, by avoiding viability loss associated with individualization (Beers et al. 2012). Similarly to other aggregate-passaging methods, EDTA passage makes precise quantification of cells more difficult, which is a serious drawback for automation and scale-up.

3.2 Culture Developments

In the two decades since hESCs were first cultured via manual passage with xeno-derived, undefined media and substrates, the field has advanced substantially to achieve fully defined, xeno-free culture with enzymatic, automation compatible passage. These advances continue, with the aim of reducing the cost, complexity and reproducibility of hPSC culture. One avenue for such progress is the removal of the necessity for substrate coating of tissue culture plastic and the treatment of cells with ROCK inhibitor at passage. Pijuan-Galito and colleagues (2016) reported that the supplementation of E8 with 50 μl/ml of the protein inter-α-inhibitor (IαI) when cells were passaged allowed for culture without prior substrate coating, or ROCK inhibition. Their data also suggested that IαI passage had a protective effect against trypsin carry-over after passage which would provide increased margin for error in an automated scale-up culture system. Similar advances could pave the way for a more robust and economically viable future for hPSC therapies. Others have

reported successful nonadherent culture (Chen et al. 2012; Steiner et al. 2010), but his has not been achieved with systematic success across the field so far.

3.3 Stem Cell Derivation for Regenerative Medicine

To ensure defined quality and safety in cell transplantation, hESCs need to be derived and maintained using good manufacturing practice (GMP) standards, set down by both the European Medicines Agency and the Food and Drug Administration. Thus, before hESC lines can be effectively deployed in regenerative medicine, a defined set of methodologies and criteria needs to be established for their safety and quality. The protocols for the establishment and in vitro culture of hESC are currently varied and have in the past necessitated the use of animal-derived products or support cells (International Stem Cell Initiative et al. 2011). Cell lines from these protocols are restricted in their usefulness in regenerative medicine, because of both the risk of introducing animal antigens and pathogens to a patient and the absence of other quality assurance measures (Martin et al. 2005; Nukaya et al. 2015). As such, only a small number of existing hESC lines are suitable for clinical use. There has been a recent push to establish cell lines using both xeno-free and GMP methodologies from the outset (Vaskova et al. 2013; Ye et al. 2017). These lines should be derived from fresh embryos, sourced from GMP standard in vitro fertilization (IVF) facilities that are surplus to patient clinical requirements (Murdoch et al. 2012). Once the ICM has been separated from the TE, cells can be initially cultured on qualified human feeder cell lines, such as human dermal fibroblast (HDF) cells, and fed with medium in which all nonhuman reagents have been replaced with xeno-free equivalents (Hewitson et al. 2016; Ilic et al. 2012). GMP-grade hESCs can be derived from the ICM of good-quality frozen IVF embryos plated onto recombinant cell extracellular matrix components such as laminin-521 and maintained in xeno-free medium (Rodin et al. 2014). There are several commonly used xeno-free culture systems that support undifferentiated growth of hPSCs consisting of a xeno-free growth medium ideally with xeno-free substratum, including TeSR2 with human recombinant laminin (LN-511), NutriStem with LN-511, RegES with human foreskin fibroblasts (HFFs), KO-SR Xeno-Free/GF cocktail with CELLstart matrix, Essential E8 (Chen et al. 2011) with recombinant vitronectin, and StemFit medium, among others. A recent study has demonstrated a fully GMP-compliant derivation system whereby fresh discarded surplus IVF embryos were cultured onto GMP-grade human feeder cells with the sequential use of commercially available media, HES-V2 and TeSR2 (Ye et al. 2017).

In order to provide cost-effective treatments, HLA-matched allogeneic tissue banks of the highest-quality clinical-grade hESCs will be required (Sect. 5.2). The UK establishment of quality standards, as well as the cataloguing and banking of high quality lines, has been greatly facilitated by national initiatives such as the human embryonic stem cell coordinators' (hESCCO) network and the UK Clinical Stem Cell Forum (Murdoch et al. 2012), which has allowed hESC derivation centers to coordinate efforts with the UK Stem Cell Bank (UKSCB – http://www.nibsc.org/

ukstemcellbank). As a result the UK has now established a bank of "ethically approved, quality-controlled stem cell lines for medical research and treatment" to help progress the dissemination and regulation of high-quality stem cells for regenerative medicine (Stacey and Hunt 2006; Geraghty et al. 2014). There are also many other national and international initiatives and companies, such as the National Stem Cell Bank in the USA, that provide PSC lines for medical research and treatment and the International Society of Stem Cell Research recently published guidelines for clinical translation (Daley et al. 2016).

4 Characteristics of hESCs and How to Assess Them

Stem cell banks such as the UK Stem Cell Bank (UKSCB) and others store, characterize, and supply ethically approved stem cells for medical research and treatment. Information about these can be found in the Human Pluripotent Stem Cell Registry (https://hpscreg.eu/). More than a thousand hESC lines have been generated worldwide (Seltmann et al. 2016). These lines exhibit a variety of differences based upon their origin, derivation protocol, passage number, and culture conditions; all these factors can impact the gene expression, epigenetics and karyotype, as well as the ability of the cells to self-renew and differentiate (Abeyta et al. 2004; Draper et al. 2004; Enver et al. 2005; Bock et al. 2011). Therefore, during hESC maintenance it is vital to regularly and comprehensively test karyotypic stability, functional pluripotency, identity, quality, safety, and suitability for intended purpose. Analyses currently performed to characterize ESCs are depicted in Fig. 3 (Singh et al. 2012; Marti et al. 2013).

Basic identification of maintenance or loss of pluripotency is possible through observation of the cells under phase contrast microscopy, to identify the characteristic morphology exhibited by pluripotent cultures. Pluripotent cell status is commonly also assessed by examining expression of self-renewal markers by immunostaining, flow cytometry, or quantitative reverse transcription polymerase chain reaction (RT-qPCR) (International Stem Cell Banking Initiative 2009; Pistollato et al. 2012; Singh et al. 2012; Marti et al. 2013; International Stem Cell Initiative et al. 2007).

Functional pluripotency is defined as the capacity for cells to differentiate into each of the three germ layers (endoderm, ectoderm, and mesoderm). While molecular expression of pluripotency-associated markers is a strong indicator of functional pluripotency, the latter can only truly be verified through assessment of three germ layer differentiation by one of the following: assessment of spontaneous differentiation, in vitro embryoid body (EB) formation (Itskovitz-Eldor et al. 2000; Peterson and Loring 2012), in vitro-directed differentiation using growth factors or small molecules, or in vivo through teratoma formation that also allows evaluation of

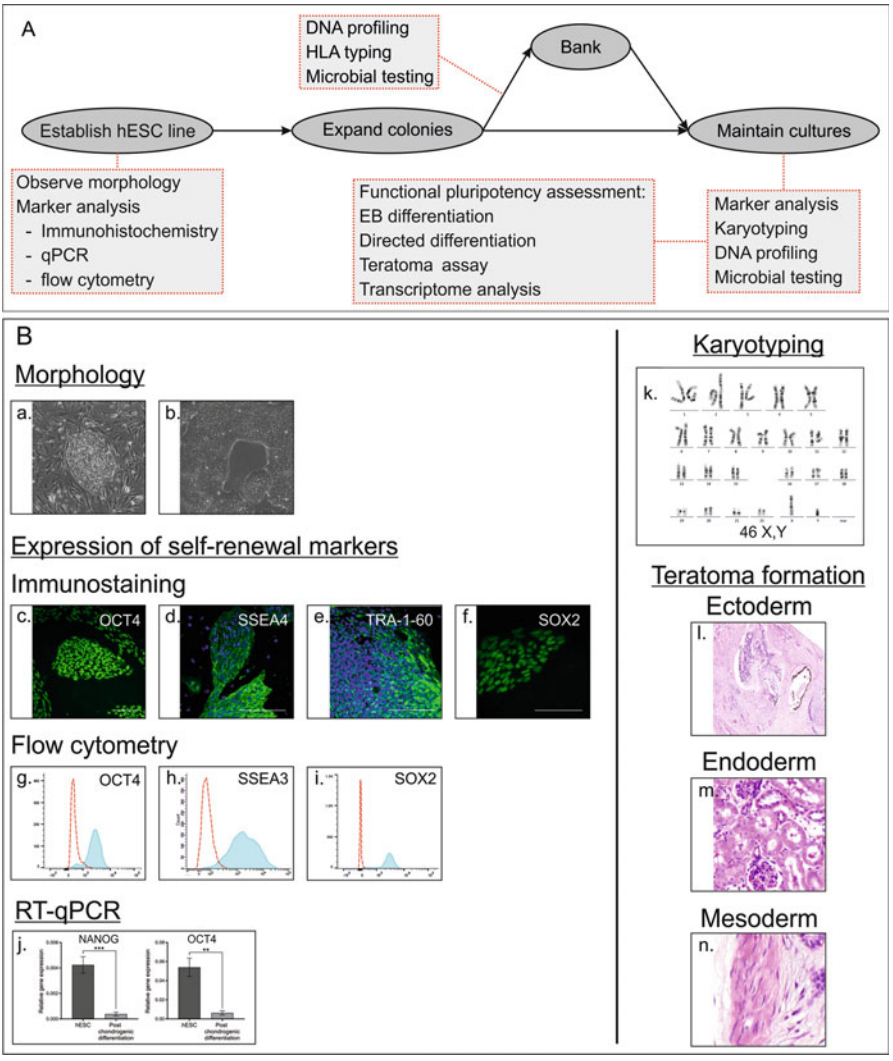


Fig. 3 Characterization of hESC lines. (**a**) Flowchart showing steps that may be undertaken when characterizing hESC lines. (**b**) Typical morphology of hESCs grown on MEFs (**a**) and vitronectin-N (**b**); immunostaining: fluorescence images of hESCs stained with OCT4 (**c**), SSEA4 (**d**), TRA-1-60 (**e**), and SOX2 (**f**) in green and DAPI in blue. Scale bars represent 100 μm; flow cytometry analysis: representative overlay histograms showing the profile of OCT4 (**g**), SSEA3 (**h**), and SOX2 (**i**) reactivity on hESCs in blue with the unstained cells in red; RT-qPCR showing relative expression of NANOG and OCT4 in hESCs and differentiated cells (**j**). Karyotyping: G-banding of hESCs showing a normal male karyotype (**k**). Hematoxylin and eosin staining of the three germ layers in a teratoma formed from a hESC line (**l**–**n**)

ability of cells to form tissues (Gertow et al. 2007). The teratoma assay (discussed further under Sect. 4.5, below) currently remains the gold standard for assessing the ability of stem cells to form tissues from all three germ layers. Stem cell biologists have recognized the need to standardize methodologies in the field, resulting in global initiatives such as the International Stem Cell Initiative (ISCI, http://www.stem-cell-forum.net/initiatives/isci).

4.1 Morphology of hESCs

An important skill in successful culturing of hESCs is the ability to recognize the morphology of undifferentiated cells under a variety of conditions. Undifferentiated hESCs and iPSCs cultured on feeders display a distinct morphology with a prominent nucleolus and a high nucleus to cytoplasm ratio. Cells are arranged in flat, tightly organized colonies with defined edges (Amit and Itskovitz-ELDOR 2012). Observing hPSC morphology is a quick and inexpensive way to assess good colonies because colonies that have started to differentiate develop rough edges with loosely organized cells. This is especially true in a research setting where the number of cell lines is small enough to be manageable. However, for scale-up, it is not feasible to examine all cells by the eye (Rosowski et al. 2015), thus generating a need for automated imaging software that can quickly and reliably examine the morphology of large numbers of PSCs (Kerz et al. 2016 ; Perestrelo et al. 2017). It may be that automated measurement of cell size, density, nucleus to nucleolus ratio, and nucleus to cytoplasm ratio will be useful metrics that can be used by imaging software to judge the quality of PSCs. However, since PSCs cultured under different conditions can have slightly different morphologies (Ludwig et al. 2006a), significant experience in inspecting PSC cultures is required, and it is important for laboratories to keep representative images of ideal colonies for each cell line and examples of poor-quality cultures that have areas of differentiation as an aide-memoire and for training purposes. In order to facilitate scale-up and medical application, feeder-free systems of culturing hESCs have been developed. In feeder-free systems, PSC colonies often appear looser soon after plating, but as the cells divide, they compact to form the typical PSC colony morphology (Healy and Ruban 2014; Vestergaard et al. 2016). This is partly a function of the absence of feeder cells but also of the manner in which these cells are often passaged. While feeder-dependent cells are typically passaged in large aggregates, feeder-free cells are more often passaged in smaller aggregates or even single cells (Sect. 3.1.3). As a result, PSCs spread more diffusely across the culture surface, migrate actively, and proliferate in the initial hours of culture and then form the more typical PSC culture morphology as their density increases.

While the assessment of PSC morphology is a valuable method of regularly observing broad colony quality trends during culture progression, proper evaluation of pluripotency requires assessment of self-renewal/pluripotency-associated markers (“Assessing Pluripotent Cells”).

4.2 Molecular Assessment of Pluripotent Cells

Many studies have utilized transcriptome analyses to compare different undifferentiated hESCs and hiPSCs to their differentiated counterparts (Sato et al. 2003; Dvash et al. 2004; Gifford et al. 2013) (Sect. 4.5.3). These studies have found that hESCs are enriched in hundreds of genes including OCT4, SOX2, NANOG, REX1, FLJ10713, DNMT3B, FOXD3, SALL2, GABRB3, and TDGF1, suggesting that they play a role in maintaining pluripotency. It is accepted that a core triad of these transcription factors regulates the pluripotent state: OCT4 (POU5F1), SOX2, and NANOG (De los Angeles et al. 2015; Li and Izpisua Belmonte 2016), but the regulation of pluripotency is not limited to protein-coding genes. Several microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are differentially enriched in PSC prior to and after differentiation and contribute to controlling stemness (Houbaviy et al. 2003; Lakshmipathy et al. 2007; Li and Izpisua Belmonte 2016). The publication of these gene expression signatures has paved the way for the development of in silico tools that predict pluripotency based on transcriptome data and have revealed potential markers that can positively identify hPSCs.

ISCI has suggested a core set of markers that play a role in pluripotency or are consistently expressed in 59 hESC lines, including NANOG, TDGF, OCT4, GABRB3, GDF3, DNMT3B, the keratan sulfate antigens Trafalgar (TRA)-1-60 and TRA-1-81, and the glycolipid antigens stage-specific embryonic antigen (SSEA)3 and SSEA4 (Draper et al. 2002; Henderson et al. 2002; Chambers et al. 2003; Sperger et al. 2003; Dvash et al. 2004; Richards et al. 2004; International Stem Cell Initiative et al. 2007). hPSCs may also be identified through the detection of SOX2, REX1, and hTERT expression, as well as alkaline phosphatase (AP) activity (Singh et al. 2012; Marti et al. 2013). As can be seen from the list above, a wide range of markers whose expression is associated with pluripotency have been used as surrogate evidence of hPSC pluripotency, but some markers are more informative than others. For instance, during the directed differentiation of hESCs into the three germ lineages, OCT4, SSEA3, and TRA-1-60 were consistently downregulated earlier than AP and NANOG (Ramirez et al. 2011). Levels of SOX2 and OCT4 expression need to be precisely regulated to maintain pluripotency, with either higher or lower expression leading to the loss of pluripotency in ESCs (Fong et al. 2008; Kopp et al. 2008).

Various methods are used to detect self-renewal markers. Live fluorescent substrate-based staining is used to detect AP activity (Singh et al. 2012; Marti et al. 2013). At the protein level, antibody-based methods such as imaging and flow cytometry are often used; immunostaining of intracellular proteins like OCT4, NANOG, and SOX2 requires cell permeabilization and the termination of the culture. Alternatively, intracellular markers can be detected in live cells through the use of molecular beacons – short single-stranded oligos that bind to complementary mRNA, allowing the attached reporter to fluoresce (Santangelo et al. 2006; King et al. 2011). A limitation of using molecular beacons is that they need to be delivered into the cells and the delivery method may be inefficient or even toxic. In addition, RT-qPCR is useful to generate quantitative or semiquantitative expression data.

It is important to keep in mind that no single marker is sufficient to identify PSCs. Many genes are expressed in multiple tissues. For instance, SOX2 is expressed in neural progenitor cells and PSCs (Graham et al. 2003). Also, pluripotency-regulating genes may have splicing variants with different functions, as illustrated by OCT4, which has an A isoform relevant to pluripotency and a B isoform that is not (Marti et al. 2013), TCF3, FOXP1, NANOG, and MBD2 (reviewed: Li and Izpisua Belmonte 2016). Moreover, several processed OCT4 pseudogenes exist in the human genome with high homology to OCT4 (Suo et al. 2005) that may cause a false-positive signal in gene expression experiments. OCT4 distal enhancer activity is considered a molecular signature of ground state (Sect. 4.3) pluripotency, a pluripotent state that may resemble the preimplantation embryonic configuration in mouse (Ying et al. 2008). However, both distal and proximal enhancer elements of OCT4 are active in naïve and primed pluripotent states and cannot be used as a binary distinguishing marker since it is their relative activity level and dominance that determine if the cells are pluripotent (Buecker and Wysocka 2012; Karwacki-Neisius et al. 2013). Therefore, it is considered good practice to carefully select a panel of markers and analyze them using validated detection methods. It is important to note that even the presence of several markers does not guarantee that a cell is a PSC; for example, OCT4, NANOG, and SOX2 must be expressed to the right level and in a specific equilibrium to maintain pluripotency (Kashyap et al. 2009). Therefore, using more markers increases the likelihood of identifying pluripotent cells accurately, and this has driven the trend toward using multigene expression panels.

Positive marker panels only confirm the presence or absence of PSCs, but they do not detect contamination by differentiated cells (Muller et al. 2008; Marti et al. 2013). Accordingly, when characterizing PSCs, it is also useful to include a selection of negative markers of early differentiation such as SSEA1, A2B5, CD56, GD2, GD3, and CD13 (Draper et al. 2002; International Stem Cell Initiative et al. 2007).

4.3 Naïve Pluripotency

Studies on murine ESCs have provided a great deal of insight and facilitated the pioneering derivation and characterization of the first hESCs. However, there are a number of profound differences between hESCs and mESCs, including the apparent different developmental status of the ICM/epiblast at the point of derivation. While both mouse ESCs and hESCs are derived from the inner cell mass of the preimplantation blastocyst, mouse ESCs are in a pluripotent state termed “naïve” or ground state pluripotency (Bar-NUR et al. 2011; Hackett and Surani 2014), while human ESC cells are in a state of pluripotency which shows similarity but not identity with that of the “primed” postimplantation mouse epiblast rather than the ICM (Brons et al. 2007; Tesar et al. 2007). Naïve pluripotent cells seem to exist transiently during mouse development, between ICM formation and their priming in preparation for lineage specification. The murine ESCs are considered to represent a developmentally “blank slate,” while their “primed” pluripotent counterparts in humans are considered to be poised for differentiation (Polo et al. 2010). This state of naïve

pluripotency is not only characterized by the expression of key pluripotency factors but critically hypomethylation and a derestricted epigenome free of developmental bias (Leitch et al. 2013).

It is not yet known whether cells of the human ICM possess the biological characteristics of naïve pluripotency during normal development. Researchers have replicated aspects of the naïve state in hESCs artificially, both through the use of transgene expression and more recently through induction by small molecules (Buecker et al. 2010; Duggal et al. 2015; Guo et al. 2016). These induced-naïve hESCs have potential to improve regenerative therapies as their open chromatin configuration increases the success rate of gene-editing technologies in these cells (Hackett and Surani 2014) which could allow gene correction for personalized therapies. Additionally, it has been reported that when iPSCs are induced to a more naïve state and directed to differentiate, the culture increases expression of desired differentiation markers compared to the iPSC parent cell line (Duggal et al. 2015). While there is potential for improvements in regenerative therapies through the induction of naïve pluripotency in hESCs, there is still a great deal of research still to be done. There are molecular and functional differences between pluripotent cell types, which subsequently influence their characteristics, function, and safety; however it remains to be elucidated whether there are drawbacks to maintaining PSCs under naïve conditions and if there are additional safety concerns associated with naïve pluripotency. For instance, it is currently unknown whether naïve stem cells have an increased tendency to acquire genomic abnormalities due to their open chromatin configuration and reduced repressive epigenetic marks (Weinberger et al. 2016).

4.4 Genomic Stability of hESCs

hESCs need to be screened carefully to rule out the occurrence of genetic abnormalities, which can arise either from the source embryonic material or occur during long-term ESC culture (International Stem Cell Initiative et al. 2011; Laurent et al. 2011). hESC lines should be screened initially since a large proportion of early human embryos are chromosomally abnormal as a result of meiotic errors arising in the gametes or post-zygotic errors arising during cleavage resulting in mosaic embryos containing both euploid and aneuploid cells (Harper et al. 2004). Ongoing screening is equally important as both pluripotency and genetic stability may change during adaptation to different passaging methods and culture conditions (Brimble et al. 2004; International Stem Cell Initiative et al. 2011). Weissbein and colleagues (2014) summarized the common genetic abnormalities in cultured PSCs in their review. These chromosomal modifications, such as those on chromosomes 1, 12, 17q, and 20, are reminiscent of those observed in cancers (for instance, chromosome 20 duplication resulting in enhanced expression of BCL2L1, a classical cancer gene). They typically accumulate by predisposing cells toward increased survival and replication, such that PSC cultures become overgrown with abnormal cells but often retain a minority of normal karyotypes (Draper et al. 2004; International Stem

Cell Initiative et al. 2011; Na et al. 2014). It is, therefore, important to continually monitor the quality of ESC lines. Tests that determine cell identity and ensure quality and safety also need to be carried out, especially if the cell lines are intended for banking or therapeutic use (Stacey 2012). These additional tests include more detailed genetic profiling, human leukocyte antigen (HLA) typing, and microbial testing (Young et al. 2010; Stacey 2011). Karyotyping will only reveal large deletions or translocations, and hence more refined analyses such as comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays may also be needed (De Sousa et al. 2009; Elliott et al. 2010; Canham et al. 2015), although complete sequencing of the genome has little clinical value at the moment. In summary, tests that confirm functional pluripotency and normal genetic status are important for documenting the characteristics of hESC lines and need to be performed periodically throughout the cell line expansion: every ten passages may be a realistic goal with repeated return to early passage stocks to avoid acquisition and enrichment of genetic changes selected through culture adaptation.

4.4.1 Epigenetic Characterization and Regulation

Pluripotent stem cells are governed by a network of transcription factors whose own transcription is regulated at the epigenetic level, notably by histone modification. In general terms the core pluripotent complex acts by repressing developmental genes, particularly though upregulation of polycomb proteins. A subset of the polycomb group (including Ezh2 as methylase) complex with the dimethylated histone 3 lysine 27 (H3K27me2) to induce trimethylation and hence repression of developmentally regulated genes (Shirane et al. 2016; Boyer et al. 2006). The trithorax proteins, particularly Wdr5, interact with H3K4me2 converting it to me3, an activating mark which is found associated with active genes in pluripotent stem cells (Ang et al. 2011). The demethylation of the inhibitory H3K9me3 mark is also involved in the maintenance of pluripotent cells (Becker et al. 2016). Additionally, histone acetylation is important, although less well documented, and histone deacetylase inhibitors have been shown to improve reprogramming from somatic cells. It has been proposed that pluripotent stem cells are poised for differentiation since they carry bivalent marks, both activating H3K4me3 and inhibiting H27K27me3 (Steward et al. 2006; Hochedlinger and Plath 2009), and this forms another very characteristic feature of hESCs. This is combined with and interacts with DNA methylation: globally DNA is hypomethylated (Hackett and Surani 2014) although the situation is far more complex with differences between pluripotent cells and somatic in the methylation at CpG islands (Lister et al. 2009) and a predominance of hydroxymethylated cytosine (Krishnakumar and Blelloch 2013).

4.5 Assessing Functional Pluripotency

To test PSC lines for pluripotency, it is important to confirm ability to give rise to cells of all three germ layers: ectoderm, mesoderm, and endoderm. In mice, it is possible to do this directly by testing chimera formation, germline transmission, and

tetraploid complementation in vivo. For ethical reasons, it is not possible to conduct similar experiments with human PSCs although injection of hESCs (and hiPSCs) into gastrula stage mouse embryos suggests some early tissue integration is possible (Mascetti and Pedersen 2016). The closest alternative to chimera assays in the hPSC field is the teratoma formation assay (Damjanov and Andrews 2016). In vitro assays for determining functional pluripotency involve spontaneous differentiation with EB formation or directed differentiation. Alternative 'omics or similar assays for determining pluripotency have been developed in recent years.

4.5.1 Teratoma Formation Assay

The teratoma assay is a qualitative assay that involves injecting PSCs most commonly under the kidney capsule or testis capsule or subcutaneously around the scapula or flank of an immune-deficient host mouse (Gertow et al. 2007). Over the weeks, the PSCs form differentiated tumor-containing cells from the different germ lineages, often forming higher-order organoids and tissues through a combination of signals from the three-dimensional environment, cell to cell interactions, and exposure to morphogens (Przyborski 2005). Much of the tissue is immature but recognizable; e.g., cartilage, bone, neuroepithelium, kidney tubules, and gastrointestinal tract tissues can be recognized (Thomson et al. 1998; Gertow et al. 2004, 2011; Damjanov and Andrew 2016). Histological analyses are performed to assist in the identification of tissues, and employment of a fetal pathologist is useful since many of the tissues are fetal-like (Lim et al. 2015). Also, donor and host cells have been known to form combined structures and histology cannot differentiate donor versus host cells. In situ hybridization, immunostaining or transcriptomic analyses need to be undertaken to definitively show that the donor hESCs are capable of forming the specific tissues of interest. Human nuclear antigen and human cadherin-1 may be used to aid donor cell identification (Vescovi et al. 1999; Heins et al. 2004). Markers commonly used to detect ectodermal tissue include class III β-tubulin (TUJ1) for neurons, keratin for keratinocytes, and dopamine beta-hydroxylase for adrenal cells. Markers used to detect mesodermal derivatives include smooth muscle actin for myocytes, cartilage matrix protein for the bone, alcian blue for the cartilage, kallikrein 1 for the kidney, Wilms tumor protein for the kidney/urogenital tract, and cardiac muscle α-actin for the heart. For endoderm tissue, α-1 antitrypsin and insulin/C-peptide, which are expressed by hepatocytes and the pancreas, respectively, are often used (Przyborski 2005).

The teratoma assay is considered the gold standard for demonstrating pluripotency of human PSCs because it shows the pluripotent lineage range under physiological conditions and actual tissue formation and has been used for the characterization of many newly derived hESC and iPSC lines (Thomson et al. 1998; Yu et al. 2007; Tannenbaum et al. 2012; Ye et al. 2017). However, less than half of the published ESC and iPSC lines have been validated using teratoma assays (Muller et al. 2010), and the published teratoma assay methods vary greatly in terms of the number and preparation of cells, the site of injection, and the length of the incubation period. Cells can respond to their direct environment, preferring to differentiate into specific lineages depending on the injection site (Miyazono et al.

1995; Wakitani et al. 2003; Gertow et al. 2004). Since the injection site, cell number, and incubation period can all influence the outcome, and results are not quantitative, teratoma assays are vulnerable to inconsistencies due to differences in protocol, making it difficult to interpret and compare published teratoma studies.

Other disadvantages of the teratoma assay are that teratoma formation is lengthy non-quantitative and time-consuming and carries the heavy cost of housing and monitoring of host mice for the duration of the experiment (Muller et al. 2008). Thus, its utility and position as a gold standard have been called into question, with many turning to lower-burden, time-saving alternatives which avoid animal use such as in vitro differentiation assays and high-throughput profiling coupled with computer predictions (Muller et al. 2011; Buta et al. 2013; Roost et al. 2015; Tsankov et al. 2015; Avior et al. 2015) (Sect. 4.5.3).

4.5.2 Embryoid Body (EB) Formation

In vitro, hESCs in suspension can undergo spontaneous differentiation by forming EBs. EBs form when PSCs grown in suspension are deprived of pluripotency signals and start to differentiate in suspended aggregates (Itskovitz-Eldor et al. 2000; Peterson and Loring 2012). Initially, PSCs form simple EBs and densely packed aggregates of hESCs, and with time these form large cystic EBs with a lumen similar to the proamniotic cavity in the murine epiblast. EBs can be formed in a number of ways (Kurosawa 2007). The simplest method for generating EBs involves scoring PSC colonies or using mild trituration to generate smaller cell aggregates and culturing them in a nonadherent polystyrene dish (Marti et al. 2013). This results in aggregates with heterogeneous shapes and sizes that lead to asynchronous differentiation and development (Bauwens et al. 2008). Alternatively, the hanging drop method that involves harvesting hESCs as a single-cell suspension in the presence of ROCK inhibitor and allowing a defined number of cells to collect and aggregate at the bottom of the drops generates EBs with homogeneous and defined sizes (Watanabe et al. 2007). EBs can also be formed through forced aggregation, where prescribed numbers of single cells are placed in round-bottom wells or triangular microwells and cells are allowed to collect through gravity or centrifugation (Ng et al. 2008; Nakazawa et al. 2013).

Fully formed EBs can be allowed to spontaneously differentiate or can be used for directed differentiation. EBs kept in suspension or transferred onto an adherent surface and grown without FGF2 over 7–21 days will differentiate spontaneously (Kaur and Tilkins 2013; Lin and Chen 2014). Thereafter, immunostaining or RT-PCR of lineage-specific markers is used to confirm tri-lineage differentiation. Commonly used markers include TUJ1 for (neur)ectoderm, α-smooth muscle actin (α-SMA) for mesoderm, and alpha-fetoprotein (AFP) or GATA4 for endoderm (Skalli et al. 1989; Katsetos et al. 2003; Kwon et al. 2006). At present, there are no standard methods for generating, differentiating, or analyzing EBs. Different methods, media compositions, and the size of EBs can all influence the differentiation trajectory (Kurosawa 2007; Bauwens et al. 2008). For instance, the forced aggregation method in microwells simulates hypoxic conditions and tends to favor cardiac differentiation (Nakazawa et al. 2013), which has been exploited to direct differentiation into cardiomyocytes (Pesl et al. 2014). EBs generated using different methods have

also been used for the directed differentiation, e.g., to pancreatic cells (Phillips et al. 2007), osteoclasts (Grigoriadis et al. 2010), cartilage (Koay et al. 2007; Toh et al. 2007; Hwang et al. 2008), skeletal muscle cells (Hwang et al. 2013), and dopaminergic neurons (Datta et al. 2013).

4.5.3 Gene Expression Approaches

The methods described in the previous three sections are time-consuming and mostly qualitative. Gene expression profiling can be performed to confirm a cell type or cell state. Transcriptome studies are generally performed using qPCR, microarrays, or RNA sequencing. Recent studies have investigated using gene expression signatures as a more quantitative, efficient way to assess the quality and potential of hPSCs, resulting in the development of novel in silico tools that can be used to characterize stem cells. Examples of these platforms are briefly described in this section: PluriTest (Muller et al. 2011), ScoreCard (Tsankov et al. 2015), TeratoScore (Avior et al. 2015), and KeyGenes (Roost et al. 2015).

The teratoma assay gives a qualitative assessment of germ layer contributions to the ES-derived tissue, but it is also possible to quantitatively assess the differentiation potential of hPSCs by evaluating the gene expression pattern in a teratoma using the TeratoScore online tool (Avior et al. 2015). PluriTest can be used to assess the pluripotency of cells with a high degree of sensitivity and specificity based on gene expression profiles (Muller et al. 2011). The ScoreCard assay can also be used to predict the in vitro lineage bias of PSC lines; it uses qPCR measurements of a set of 96 genes to evaluate the molecular signature of pluripotency and expression signatures that indicate functional pluripotency (Tsankov et al. 2015). The KeyGenes algorithm evaluates tissue differentiation efficiency in vitro and can be used to assign developmental stages to differentiated hPSC derivatives (Roost et al. 2015). None of these technologies have yet become a "standard" method of characterizing pluripotency, either by research labs or across different stem cell banks, and currently standard metrics for pluripotency are typically antibody-based assays which vary significantly lab to lab ("Assessing Pluripotent Cells").

5 Alternative Sources of Cells for Regenerative Medicine

Although hESCs are the only cell lines which are pluripotent from their initial derivation, other pluripotent stem cell lines are important in regenerative medicine and disease modeling, such as iPSCs and the multipotent mesenchymal stem cells (MSCs). Though each of these cell types is applicable in regenerative medicine, for the purposes of this chapter, we will focus on the relative advantages and disadvantages in the application of hESCs and iPSCs.

5.1 Induced Pluripotent Stem Cells (iPSCs)

Although pluripotency is only naturally present in embryonic stem cells, both multipotent stem cells and terminally differentiated somatic cells can be returned

to the pluripotent state through reprogramming, either by the induced expression of transcription factors or by treatment with small molecules ("IPSC Book Chapter"). iPSCs therefore offer an excellent method of achieving the developmental potential of hESCs but without the prerequisite of having to acquire these cells from blastocyst-stage embryos.

Since the discovery of inducible pluripotency via expression of the 'Yamanaka transcription factors' (Oct4, Sox2, Klf-4, cMyc), much work has been done to establish the functional and molecular differences between hESCs and iPSCs (Takahashi and Yamanaka 2006). All pluripotent stem cell lines are, by definition, able to generate cells of the three germ layers; however it has been demonstrated that different hESC and iPSC cell lines may exhibit biases in their ability to form different tissue types (Mehta et al. 2010). This has been demonstrated both in the relative proportions of each lineage produced after differentiation in unbiased EB or teratoma experiments and also in their relative efficiency at forming cell types by directed differentiation (Mikkola et al. 2006; Burridge et al. 2007; Kim et al. 2010) although the diverse genetic background of these cells needs to be taken into account.

During the course of development from pluripotent hESCs and ICM cells to terminally differentiated somatic cells, major changes in the epigenetic signature of these cells take place in order to permit or restrict transcription of genes relevant to each cell type. When somatic cells are reprogrammed by transcription factors, this epigenetic architecture has to be overhauled in order to reestablish pluripotency (Maherali et al. 2007); however, cells retain a considerable amount of the genomic methylation profile of their tissue of origin. This carry-over of somatic methylation profiles into the resulting iPSC has been termed "epigenetic carry-over" and leads to detectible differences in transcription, resulting in observable differences in functional pluripotency (Kim et al. 2010; Ohi et al. 2011). Indeed, it has been demonstrated that iPSCs more readily differentiate back into their tissue of somatic origin than to other tissues (Kim et al. 2010). Interestingly the carry-over of somatic cell of origin-memory in iPSC cell lines has been shown to diminish with increased passage number (Kim et al. 2010; Polo et al. 2010). One study demonstrated that at passage four, the transcriptome profile of iPSCs clearly clustered by their somatic origin; however by passage 16 these same cell lines were transcriptionally indistinguishable (Kim et al. 2010).

This epigenetic carry-over is not necessarily a bad thing for regenerative medicine but has consequences for the application of iPSCs. While the ideal iPSCs would be functionally indistinguishable from hESC cell lines, biased iPSC lines from different somatic origins could be employed to increase the efficiency of generating certain cell types, particularly those that are currently proving more challenging to achieve. Unfortunately for both regenerative medicine and in vitro disease modeling, the necessity to increase passage number to overcome this bias only adds duration to an already long process of generating lines. It may also lead to an increased likelihood of culture adaptation and/or selection of advantageous adapted karyotypic variants (Gokhale et al. 2015; International Stem Cell Initiative et al. 2011).

While both hESCs and hiPSCs have great value in regenerative medicine applications, there are advantages and disadvantages associated with each cell type, as illustrated in the succeeding table. The choice between hESCs and hiPSCs will also depend on whether autologous or allogeneic cells are needed, the former only being available for patient iPSCs. As hiPSC reprogramming takes weeks, existing cells made from HLA-matched clinical-grade hESCs may be better utilized, for acute applications such as a heart attack or skin burns. For chronic conditions such as diabetes, where time is less intensive, there may be a benefit in deriving hiPSCs from a patient and utilizing these for therapies, as they should be fully histocompatible. On the other hand, if such chronic diseases are of genetic origin, then gene correction of patient iPSCs would be needed, increasing the risks in culture time and cost. Patient-specific therapies of this type will present challenges to current models of healthcare funding.

	hESCs	hiPSCs
Advantages	Low cost	Easy to obtain
	Well established and characterized	HLA histocompatibility
	Realistic HLA spectrum	Disease modeling possible
	MHC downregulation possible	Drug/toxicity profiles possible
		Autologous use possible
Disadvantages	Immunosuppressants may be needed	Cost of reprogramming cells for individualized therapies
	Tissue rejection	Retroviral gene delivery
	Carcinogen risk	Oncogene activation risk
		Mutagenesis risk
		Retention of alterations
		Inability (time and expense) to characterize patient-specific hiPSC lines as fully as a banked HLA-matched line

5.2 Histocompatibility

A major issue for the use of hPSCs in regenerative medicine is histocompatibility. Unless a transplant is tissue matched to the patient, the use of antirejection medications is necessary to prevent the transplanted tissues from being rejected by the host immune system, as used for many years in organ transplant programs. iPSC and MSC lines can be readily generated from a particular patient and as such can theoretically be derived, expanded, and differentiated into the desired cell or tissue type and then reintroduced into the same patient. This methodology has outstanding potential for the field of regenerative medicine as such personalized therapies would drastically reduce the risk of rejection. Personalized hESC lines can also be derived,

by transferring somatic cell nuclei from a patient into an enucleated donor oocyte (known as somatic cell nuclear transfer or SCNT) which reprograms the nucleus to develop a blastocyst with a conventional ICM from which a hESC line genetically identical to the somatic nucleus can be derived (Wolf et al. 2016; Tachibana et al. 2013). Up until now the supply of donated oocytes has limited serious consideration of this method for application in regenerative medicine, but the recent demonstration that mouse ESCs and iPSCs can be differentiated into functional oocytes (Hikabe et al. 2016) may change this in the future should the technology prove transferable to human oocytes.

While promising, individualized therapies come with the caveat of having to successfully derive and rigorously quality- control several iPSC lines per patient to confirm that the cells are high quality, safe, and functionally viable. Such personalized iPSC therapies would therefore always come at an increased cost and time required to produce clinically relevant cell numbers of high-quality cells compared to being able to utilize a central bank of rigorously screened and characterized lines.

A variety of methods have been investigated to attempt to avoid the logistical issues caused by generating personalized stem cell lines for each patient. Immunosuppression has been used widely for transplantation for decades; however, as immunosuppression is both a nonspecific and long-term therapy, it is associated with an increased risk of both opportunistic infections and of malignancies overcoming a weakened immune system. Additionally, immunosuppression is not a guarantee of long-term success, and the proportion of transplantations which eventually fail despite immunosuppression increases with the degree of HLA mismatch between patient and donor (Williams et al. 2016). It is also notable that hESC-derived retinal pigment epithelium (RPE) cells have been transplanted safely and successfully without concern for histocompatibility and without immunosuppression (Schwartz et al. 2015). Healthy RPE is typically considered to be immune-privileged; however whether this privilege extends to the diseased conditions in which RPE transplantation is necessary, or indeed when the retinal barrier is breached during RPE cell transplantation, is a subject of debate (Whiting et al. 2015).

A possible solution to this problem of histocompatibility lies in generating a pool of high-quality stem cell lines which are compatible with a vast majority of the population. It has been estimated, and later demonstrated, that a PSC bank of only 150 selected homozygous HLA-typed cell lines would be required to HLA match the 93% of the UK population with zero HLA mismatch (Taylor et al. 2005, 2012). By collecting, HLA typing, expanding, and quality controlling this limited number of cell lines with a suitably immunologically diverse range of HLA types (Solomon et al. 2015), it would be possible to avoid the increased costs of personalized therapies while still reaping the benefits of HLA-matched transplantation.

Alternatively the issue of HLA matching could be bypassed entirely by generation of HLA universal cells. It has recently been postulated that by silencing expression of major histocompatibility complexes I and II (MHCI and II, the major proteins which specify immune compatibility) in pluripotent stem cells, the regenerative medicine cell products could be made to be “HLA universal,” that is,

able to be transplanted into any host without the risk of host rejection (Figueiredo and Blasczyk 2015). An excellent test case for this technology is in the mass production of HLA-universal platelets. After repeated platelet transfusions, patients can develop anti-HLA antibodies which result in an insufficient platelet count after transfusion (Schiffer 2001). As demonstrated in mouse models by Borger and colleagues (2016), the differentiation of megakaryocytes from iPSCs in which HLA Class I has been silenced allows for the development of functional HLA-universal platelets which can bypass the problem of immune action against transfused platelets. Problematically, complete silencing of the HLA Class 1 in cells leaves them vulnerable to the innate immune response of natural killer (NK) cells. Some groups are endeavoring to overcome this problem by forcing expression of minimally polymorphic HLA-E molecules, an approach which has been demonstrated to be highly successful by Gornalusse and colleagues (2017). Even if further advancements in this method prove successful, there are a variety of stumbling blocks to overcome especially as other antigens are involved in rejection, not just the MHC complex (French et al. 2015). However, there remains potential for the development of universal donor hESC cell lines which, after thorough quality control and safety assessments, could circumvent the limitations of individualized therapies utilizing iPSCs and MSCs. High-quality HLA universal hESCs could be produced en mass, to be differentiated into the tissues required for therapies at a fraction of the cost of doing so for individual patients. Some groups are endeavoring to generate universally compatible PSC lines which would not be recognized by the immune system (e.g., Gornalusse et al. 2017) to solve this problem.

6 Translating ES Cells for Therapy

6.1 Scaled-Up Culture of hESCs

In order to use hESCs for regenerative medicine, it will be necessary to generate a significant number of cells, depending on application. For some applications, for example, for blood cell transplantations, as many as 10^8 healthy, pluripotent stem cells may be required, which is a considerable scale-up from a typical six-well plate culture of 10^6 cells. For other applications the numbers needed are significantly smaller, e.g., for the recent hESC-retinal pigment epithelium, as few as 5–15 $\times$ 10^4 cells were used per eye (Schwartz et al. 2015). Any viable culture system for hESC scale-up must have fully defined, xeno-free, cost-effective substrate and medium which permits a method of culture and passage easily performed by a specialized robot. Additionally, in-line characterization of the health and pluripotency of cells would be ideal in order to ensure the quality of the cells.

At the time of writing, the most promising medium/substrate combination for this purpose appears to be E8/VTN-N (Sect. 3). This combination has been made available commercially by two independent companies (Life Technologies, California, USA; STEMCELL Technologies, Vancouver, Canada) and is in wide use within the scientific community. Cells cultured adherently in E8/VTN can be passaged

either in aggregates or in single cells, with the caveat that hESCs passaged as single cells in E8/VTN require a 24-h incubation in ROCK inhibitor at plating in order to maintain viability (Wang et al. 2013). E8/VTN-N has also demonstrated success in stirred microcarrier culture systems which are another potential avenue for increasing the efficiency of pluripotent stem cell culture scale-up (Badenes et al. 2016).

Several groups have demonstrated successful expansion of hPSCs in stirred-tank bioreactors either as aggregates in suspension or as adhered colonies on different types of microcarriers (Serra et al. 2010; Krawetz et al. 2010; Almutawaa et al. 2016; Kehoe et al. 2010; Chen et al. 2012). This technology may offer advantages in process scale-up over the standard two-dimensional static culture by providing a high surface area/volume ratio in a more controlled and homogeneous media microenvironment.

6.2 Automation of hESC Scale-Up

Automation of hESC culture is a crucial step in increasing efficiency of scale-up, bringing down costs while retaining or improving reproducibility and quality of cultures. A variety of cell culture robots are currently available commercially TAPBiosystems (n.d., TECAN), and companies interested in being part of the stem cell manufacture industry are pursuing their continued innovation and improvement. An ideal robot specifically for hESC scale-up in a closed system free of the risk of contamination would be able to:

- Feed cells.
- Assess the pluripotent status and health of cells.
- Assess whether cells require passage.
- Passage cells.
- Quantify passaged cell number and viability.
- Seed cells in fresh culture vessels.

Through the efforts of groups such as the AUTOSTEM consortium (AUTOSTEM – http://www.autostem2020.eu/), it is hoped that scientists will soon be able to take a sample of somatic tissue and derive and manufacture clinically relevant numbers of iPSCs without any human contact in an efficient, semiautomated manner ("IPSC Section/Chapter").

6.3 Assessing Pluripotency and Health of Cells

Methods such as immunocytochemistry, flow cytometry, and RT-PCR are well-established, and robust ways of assessing cell pluripotency and health in manual culture (see above) are already available as semiautomated systems. These methods of assessment, however, require all of the cultured cells to be passaged and a proportion to be removed and destroyed, decreasing the efficiency of scale-up.

Additionally, these methods only identify molecular markers associated with pluripotency, rather than functional pluripotency (evidenced by ability to differentiate into cells of all three germ layers). Offline methods such as teratoma formation or molecular array methods are still needed. In vitro techniques such as automated EB formation and directed differentiation are being developed by multiple laboratories to attempt to establish industry-accepted protocols and quantification techniques to confirm functional pluripotency. Directed differentiation has great potential as an upscalable functional pluripotency assessment technique. With the recent development of commercial kits, for instance, the STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies Inc., Vancouver, Canada), it seems likely that this approach will become standard to assess functional pluripotency of lines before and during scale-up.

6.4 Cell Passage

The most complex aspect of hESC culture to automate is culture passage, both the act of passaging and seeding cells and identifying when cells are ready to passage. Automated passage via trypsin dissociation has the benefit of allowing for rapid, accurate quantification of cell number and viability, allowing cells to be seeded with minimal cell or reagent wastage. Trypsin dissociation is also easily mechanized as protocols are simple and reproducible, and dissociation requires no physical contact with cells. However, single-cell passage remains stressful for cells requiring short-term ROCK inhibitor supplementation to prevent large-scale cell death. Long-term passage in E8:VTN with ROCK inhibitor supplementation only during passage has not been shown to cause karyotypic abnormalities or loss of pluripotency, but the goal is still to remove ROCK inhibitor from stem cell culture protocols and reduce cell stress during passage.

During routine culture of stem cells, pluripotency, cell health, and density are assessed daily via phase contrast microscopy. Trying to quantitate and automate the subjective assessments stem cell scientists make when assessing their cells in this way is a challenging proposition (Sect. 4.1).

6.5 Directed Differentiation

It is possible to mimic the signals that cells receive during successive stages of development, starting from the initial specification of ESCs or iPSCs into one of the embryonic germ lineages, followed by specification and patterning (Cohen and Melton 2011). Understanding and triggering the signaling pathways necessary and sufficient for induction of specific cell types in hESCs is a critical goal in cell regeneration therapy. Although the relevant signaling pathways to manipulate in vitro can be gleaned from developmental studies, directing differentiation to specific cells has proved challenging as it requires considerable optimization of the precise concentrations, timing, and combinations of growth factors and small molecules.

Numerous protocols for differentiating cells from progenitors of each of the three germ layers have been published in recent years. These include ectodermal lineages such as motor neurons (Wichterle et al. 2002), cerebral cortex neurons (Shi et al. 2012), forebrain interneurons (Liu et al. 2013; Nicholas et al. 2013), and cortical interneurons (Maroof et al. 2013); mesodermal lineages such as chondrocytes (Oldershaw et al. 2010), cardiomyocytes (Burridge and Zambidis 2013; Lian et al. 2013), and renal cells (Xia et al. 2013; Takasato et al. 2014); and endodermal lineages such as hepatocytes (Cai et al. 2007; Roelandt et al. 2010), intestinal cells (Spence et al. 2011), lung cells (Kadzik and Morrisey 2012; Huang et al. 2014), foregut epithelia (Kearns et al. 2013), and pancreatic cells (Van Hoof and LIKU 2013). Cell biologists are also interacting with tissue engineers to generate culture systems that will more accurately mimic important three-dimensional aspects of organogenesis. Indeed, some laboratories have taken the PSC differentiation process a step further to generate organoids or other three-dimensional structures that mimic human tissues (Mccracken et al. 2011; Spence et al. 2011; Lancaster et al. 2013; Tieng et al. 2014; Sinagoga and Wells 2015).

The starting material for directed differentiation may be cell aggregates similar to those used during spontaneous differentiation or PSC monolayers that are technically simpler to generate. During early development the pluripotent cells of the ICM differentiate during gastrulation, resulting in the formation of ectoderm, mesoderm, and endoderm progenitors. Many directed differentiation protocols mimic this using growth factors or small molecules to induce the conversion of ESCs or iPSCs into cells of the appropriate progenitor that gives rise to the desired cell type.

Developmental studies have revealed that variations in the signal intensity of members of signaling molecule families are required in the establishment and patterning of the germ layers in vivo: transforming growth factor-β (TGFβ) superfamily (including activin/nodal and bone morphogenetic proteins, BMPs) (Moustakas and Heldin 2009), the WNT family (Rao and Kuhl 2010), and fibroblast growth factors (FGFs) (Turner and Grose 2010). However, since the precise concentration of each factor to which a developing cell is exposed in the human embryo and the period of exposure are unknown, each candidate factor has to be tested at various concentrations and applied for various durations. For hESCs, transient Wnt followed by lower concentrations of the TGFβ family member activin A induce mesoderm lineage initiation (Yang et al. 2008; Oldershaw et al. 2010), whereas higher activin A concentrations induce endoderm formation (Kroon et al. 2008; Vallier et al. 2004). BMP4 is also required for efficient mesoderm induction (Yang et al. 2008). Noggin and DKK1, or small-molecule antagonists of endogenous BMP and WNT signaling, respectively, are used for ectoderm induction of hESCs (Lamba et al. 2006; Oshima et al. 2010; Efthymiou et al. 2014).

Following successful induction of germ layer progenitors, growth factors and signaling molecules, often in combination with small molecules, have been used to direct the cells further along the desired differentiation pathway. Researchers exploit the fact that similar signaling pathways are used at different stages and for different purposes during development. By sequential use of combinations of signaling molecules with titration of concentration and duration of application, a wide variety

of cell fates can be generated. Recent research indicates that early temporally refined stimulation of individual selected growth factor pathways (TGFβ, BMP, FGF, Wnt, and Hedgehog) combined with strategic pathway inhibition to remove unwanted lineages regulates binary switches in lineage commitment and can direct to specific mesodermal lineages in 3–5 days (Loh et al. 2016).

Recombinant factors are often produced in engineered bacterial or mammalian cells, traces of which may contaminate the final preparation, thus preventing their use in clinical application. Furthermore, the high cost of recombinant growth factors may limit their application in larger-scale differentiation procedures. Small molecules which activate or block signaling pathways offer an attractive alternative; they are more stable, less expensive, have less lot-to-lot variability, and are generally non-immunogenic. Hence, cells differentiated using small molecules might be more suitable to therapeutic transplantation than those treated with recombinant proteins (Ding and Schultz 2004; Rubin 2008). As a result, high-throughput chemical screening approaches have been used to identify novel molecules that produce the desired effect without the use of growth factors (Ding and Schultz 2004). An example of such a screen that aimed to identify small-molecule inducers of endoderm to replace activin A produced two molecules termed inducer of endoderm-1 and endoderm-2, both of which induced endoderm more robustly than activin A treatment by activating the TGFβ signaling pathway (Borowiak et al. 2009). However it is unlikely that all components of a differentiation pathway will be able to be replaced by small molecules.

Small-molecule agonists and antagonists of the Hedgehog pathway have proved very effective for motor neuron differentiation (Frank-Kamenetsky et al. 2002; Wichterle et al. 2002). Similarly, SB-431542, which antagonizes the nodal receptors ALK4, ALK5, and ALK7 (Laping et al. 2002), can substitute for protein antagonists of TGFβ in the differentiation of neurons and hepatocytes from hESCs (Smith et al. 2008; Chambers et al. 2009; Touboul et al. 2010). Some small molecules, such as KAAD-cyclopamine (D'Amour et al. 2006; Chen et al. 2009) and SU5402 (Oshima et al. 2010; Turner and Grose 2010), inhibit signaling through pathways for which an endogenous inhibitor is not known (Hedgehog and FGF signaling, respectively). Endogenous small molecules with roles in embryonic development may also be used in vitro to induce differentiation. For example, retinoic acid, a morphogen that is important in the patterning of the central nervous system in vivo (Oshima et al. 2010), has been used successfully to generate retinal cells from hESCs (Osakada et al. 2009). Similarly, the naturally occurring small molecule taurine has been used to direct the differentiation of retinal cells (Osakada et al. 2009).

Many directed differentiation protocols have a relatively low efficiency of desired cell generation, and there are safety and cost concerns posed by the reagents used to direct cell fate. It is important that hESC-derived progeny are functionally mature. It has been shown that differentiated products of hESCs and iPSCs retain an immature phenotype even when apparently terminally differentiated (Patterson et al. 2012). Although it is critical to find the appropriate culture conditions and microenvironments to ensure sufficient maturity and functionality of hESC-derived progeny, some plasticity may actually be an advantage for adaptation and continued differentiation

of transplanted cells ("Translation"). Thus, regardless of the protocol used for directed differentiation, it is necessary to monitor the efficiency, specificity, and functional maturity achieved by the various differentiation protocols. Finally, demonstrating that cells produced in vitro are functionally equivalent to those produced in vivo remains a challenging but essential element of any directed differentiation protocol.

Extracellular matrix-mediated signals include mechanical stimuli such as strain, shear stress, substrate rigidity, and topography, all of which have an impact on stem cell phenotype (Kshitiz et al. 2012). Therefore, the appropriate culture environment includes both precisely controlled biochemical and biophysical signals, some of which are provided by 2D or 3D matrices, to guide hESC differentiation toward specialized cells and development of functional tissue substitutes. For instance, substrate stiffness and application of uniaxial strain affect differentiation of mesenchymal stem cells down to osteogenic and chondrogenic lineages (Engler et al. 2006; Kurpinski et al. 2006), suggesting that the biophysical environment would also play a role in the production of osteoblasts and chondrocytes from hESCs. For bone tissue engineering, osteogenic cells are combined with biomaterial scaffolds and signaling molecules and, in some cases, subjected to dynamic in vitro culture in bioreactors in order to construct three-dimensional bone substitutes (De Peppo and MAROLT 2013; Curtis and Riehle 2001).

6.6 Directing Differentiation to Specific Lineages

Due to space constraints, it is not possible to detail here all of the directed differentiation protocols in the literature. Instead, we highlight some of the most successful protocols that have been developed so far, using one example cell type for each of the germ layers: dopaminergic neurons (ectoderm), pancreatic beta cells (endoderm), and chondrocytes (mesoderm), with an example of a directed differentiation protocol for each of these shown in Fig. 4.

6.6.1 Generation of Dopaminergic Neurons from hESCs

Stem cell therapy is being explored as a clinically viable treatment option for a number of CNS disorders (Bjorklund and Lindvall 2000). Here we summarize the work undertaken to generate dopaminergic neurons for cell replacement therapy for Parkinson's disease (PD). PD is a neurodegenerative disorder caused by decreased stimulation of the motor cortex due to progressive degeneration of dopaminergic neurons of the substantia nigra, leading to motor dysfunction characterized by muscle rigidity, tremor, bradykinesia, and akinesia (Kalia and Lang 2015). Clinical improvements have been reported following the transplantation of fetal mesencephalic grafts into PD patients (Lindvall and Bjorklund 2004; Barker et al. 2013; Kefalopoulou et al. 2014). However, several ethical and logistical issues with the use

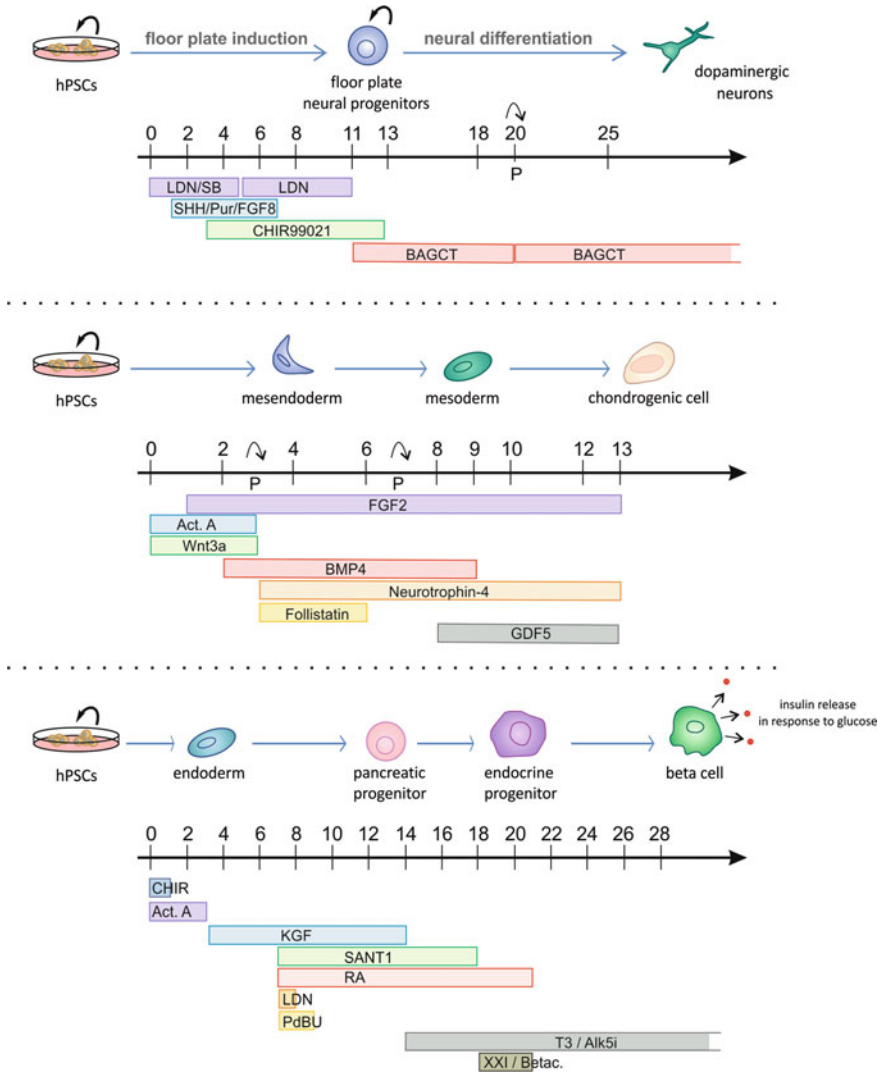


Fig. 4 Examples of directed differentiation protocols for dopaminergic neurons (Kriks et al. 2011), chondrogenic progenitors (Oldershaw et al. 2010), and pancreatic beta cells (Pagliuca et al. 2014). *LDN* LDN193189, *SB* SB431542, *SHH* purmorphamine + SHH C25II, *BAGCT* BDNF + ascorbic acid + GDNF + dbcAMP + TGFβ3, *FGF2* basic fibroblast growth factor, *Act. A* activin A, *BMP* bone morphogenetic protein 4, *GDF5* growth/differentiation factor 5, *CHIR* CHIR99021, *KGF* keratinocyte growth factor, *RA* retinoic acid, *SANT1* Sonic Hedgehog pathway antagonist, *PdbU* phorbol 12,13-dibutyrate, *Alk5i* Alk5 receptor inhibitor II, T3, triiodothyronine, *XXI* g-secretase inhibitor, *Betac* betacellulin

of aborted fetal tissue limit this approach. hESCs may provide an alternative source of dopaminergic neurons for transplantation (Kim et al. 2002; Perrier et al. 2004; Lindvall and Kokaia 2009; Barker 2014). Using protocols based on extrinsic patterning cues that mimic fetal midbrain development, it is now possible to generate dopaminergic neurons with an authentic midbrain phenotype from human PSCs that survive transplantation and can restore motor deficits in animal models of PD (Kriks et al. 2011; Kirkeby et al. 2012; Grealish et al. 2014).

Early studies showed that through the combined use of FGF8 and Sonic Hedgehog, hESC-derived neural progenitors could be differentiated in vitro into dopamine neuron-like cells expressing tyrosine hydroxylase, which is required for dopamine synthesis, and transcription factors such as Pax2, Pax5, and En1 that are indicative of dopamine synthesis (Perrier et al. 2004; Yan et al. 2005; Martinat et al. 2006), in addition to demonstrating the electrophysiological properties of midbrain dopamine neurons (Kim et al. 2002, 2007). However, most of the early protocols included undefined reagents due to co-culture with stromal cell lines or the use of conditioned media, thus limiting their translational potential. As a result, subsequent studies focused on generating dopaminergic neurons from hESCs using completely defined factors (Lukovic et al. 2017 SCTM).

Human ESCs can be differentiated into tri-lineage neural progenitors that are capable of giving rise to neurons, astrocytes, and oligodendrocytes by growing them as neurospheres in the presence of EGF and FGF2 (Joannides et al. 2007). A commonly used protocol for inducing PAX6+ neural ectoderm with >80% efficiency involves dual-SMAD inhibition, inhibiting the BMP and TGFβ pathways in a PSC monolayer using Noggin and the small molecule SB431542 (Chambers et al. 2009).

The midbrain floorplate contains A9 dopaminergic neurons that are lost in PD. In the developing midbrain, the floor plate marker FOXA2 and the roof plate marker LMX1A are co-expressed. Floor plate precursors have been derived from hESCs (Fasano et al. 2010) using a modified dual-SMAD inhibition protocol (Chambers et al. 2009). To induce LMX1A expression in FOXA2+ floorplate precursors derived from hPSC, Kriks et al. utilized small-molecule activators of Sonic Hedgehog and canonical WNT signaling using a time-specific application of GSK3β inhibitor CHIR99021 (Kriks et al. 2011). This induced LMX1A expression and neurogenic conversion of hPSC-derived midbrain FOXA2+ floorplate precursors toward dopaminergic neuron fate. Following floor plate induction, further maturation was carried out in Neurobasal/B27 medium supplemented with ascorbate, BDNF, GDNF, TGFβ3, and dbcAMP, generating engraftable midbrain DA neurons by day 25 that could be maintained in vitro for several months (Kriks et al. 2011).

Although much progress has been made in generating human dopaminergic neurons, several issues remain to be addressed before stem cell-based therapies will be feasible for PD. Differentiation protocols need to be further optimized to reliably produce pure and homogeneous populations of midbrain dopaminergic neurons that survive, reinnervate, and restore dopaminergic neurotransmission in the striatum. Long-term efficacy in vivo and safety of the procedure need to be fully assessed. Furthermore, patient selection, postoperative rehabilitation, and immunosuppression need to be optimized to maximize therapeutic benefits.

6.6.2 Generation of Pancreatic Beta Cells from hESCs (Endoderm)

Pancreatic beta cells regulate metabolic homeostasis by sensing glucose and producing the glucose-lowering hormone insulin. Type 1 diabetes mellitus is a metabolic disease in which the loss of pancreatic beta cells results in elevated blood glucose levels. Currently, type 1 diabetes is managed through daily injection of insulin (or through insulin pumps) matched to meals; however exogenous insulin replacement does not adequately control glucose levels, resulting in complications such as retinopathy, nephropathy, neuropathy, and increased risk of cardiovascular disease.

In this section we highlight recent progress in directed differentiation of hESCs toward a beta cell fate. Human PSCs, with their vast expansion capacity and differentiation potential, might provide sufficient fully functional insulin-secreting beta cells to treat diabetes. Importantly, replacement can occur away from the pancreas, meaning that cells could be transplanted subcutaneously in minimally invasive surgeries.

Directed differentiation studies have attempted to mimic pancreatic islet cell development, first focusing on the production of pancreatic and duodenal homeobox 1 (PDX1)-positive pancreatic endoderm with increasing use of defined culture conditions in later studies (Pagliuca and Melton 2013). The first step in beta cell generation is endodermal specification (D'Amour et al. 2005). After endodermal specification, signals from adjacent developing tissues induce specification of pancreatic progenitors that have the potential to generate all three pancreatic cell types: ductal, acinar, and endocrine. After selection of endocrine fate, endocrine progenitors are specified to become insulin-producing cells.

Several protocols generated beta cells that were responsive to direct depolarization, but not to high glucose concentrations (Basford et al. 2012; Takeuchi et al. 2014; D'Amour et al. 2006) meaning that these cells lacked the coupling of glucose influx to electrical activity. Similar protocols were developed to enhance differentiation into PDX1+ and Nkx6.1+ pancreatic progenitors through the addition of BMP inhibitors and protein kinase C (PKC) activators (Kroon et al. 2008; Chen et al. 2009; Nostro et al. 2011; Rezania et al. 2012). With the idea that the final stages of differentiation could be completed naturally in vivo, several groups pursued a strategy of transplanting PDX1-positive progenitor cells. One of the key advances in this field was the demonstration that the pancreatic progenitors generated can further differentiate into functional, glucose-responsive β-cells that co-express insulin and key transcription factors (PDX1, NKX6.1, and MAFA) in vivo when transplanted into a mouse and are sufficient to restore regular glucose levels in murine models of diabetes (Kroon et al. 2008; Rezania et al. 2012).

These promising data paved the way for a clinical trial testing the approach of progenitor cell transplantation (Sect. 6.7). In 2014, ViaCyte received approval for a phase I/II trial (NCT02239354) to test the safety, cell survival, and insulin secretion of a product that combines progenitor cells (PEC-01) and a macro-encapsulation device called Encaptra (Schulz 2015). Encaptra is a device that encapsulates the implanted cells, protecting them from a patient's alloimmunity and autoimmunity, while allowing oxygen, nutrients, and proteins to freely transport across the system's

membrane, thus allowing the implanted cells to sense glucose in the bloodstream and release insulin accordingly.

Recently, researchers recorded the first cellular-level evidence of glucose responsiveness, albeit at a superphysiological level, in stem cell-derived beta cells (Rezania et al. 2014). However, these cells generally failed to inactivate their calcium responses, which is an essential feature of mature beta cells that prevents hypoglycemia in the postprandial state. A similar protocol was published producing functional beta cells in vitro termed SC-β (Pagliuca et al. 2014). Importantly, glucose-responsive insulin secretion was reported from 2 weeks after transplantation into immunocompromised mice. A 2016 follow-up paper reported that encapsulated SC-β cells could reverse hyperglycemia in streptozotocin-injected C57BL/6 mice (Vegas et al. 2016).

These recent publications provide the first convincing evidence of glucose responsiveness from hESC-derived beta cells in vitro *and* in vivo (Pagliuca et al. 2014; Rezania et al. 2014; Vegas et al. 2016). Exciting progress has therefore been made toward the ultimate aim of creating fully functional β-cells from pluripotent cells. However, additional studies are required to convincingly show that these are fully functional, responding to glucose in a quantitatively similar manner to healthy human primary cells. Moreover, the different assessments of beta cell functionality, showing dynamic insulin secretion in response to multiple stimuli, need to be standardized to allow direct comparison of differentiation protocols. Finally, even after fully functional pancreatic beta cells are produced, it remains to be determined whether encapsulation can protect these cells from recurrent autoimmune attack in people with type 1 diabetes (Vendrame et al. 2010; Schulz 2015).

6.6.3 Generation of Chondrocytes from hESCs (Mesoderm)

Hyaline articular cartilage is an avascular tissue that lines synovial joints, allowing bones to glide over each other with little friction. Due to its avascular nature, articular cartilage has a low intrinsic capacity to repair itself. Articular chondral lesions are a major risk factor for the development of osteoarthritis (OA), a common and debilitating joint disease (Goldring and Goldring 2007). The current treatment option for OA that reduces pain and improves function is total joint arthroplasty; however, this is not a suitable treatment option for younger patients since they would outlive their implant and require multiple surgeries (Widuchowski et al. 2007; Jones and Pohar 2012; Aggarwal et al. 2014). Guided by knowledge of development, and physiology of native cartilage, tissue engineering efforts have sought to address this problem through the generation of chondrogenic cells/tissue that can be implanted to replace damaged tissue in order to treat symptomatic patients. The aim is to circumvent the onset of OA among individuals with small lesions, so predisposed to developing it, or repair full-blown cartilage lesions. Although multiple adult cell-based strategies have been attempted to restore joint cartilage and prevent progressive degeneration, the formation and long-term maintenance of permanent hyaline cartilage have not yet been achieved (Hunziker et al. 2015).

One of the major research goals for tissue engineering for cartilage regeneration is to expand chondroprogenitors to yield large numbers without the loss of chondrogenic activity. Because of their unlimited proliferative capacity and

pluripotency, human PSCs are a suitable alternative source. Here we focus on the recent progress using development-informed paradigms to control the differentiation of hESCs to an articular chondrocyte fate.

The process of epithelial-to-mesenchymal transition mediated by recombinant activin A, BMP4, VEGF, and FGF2 treatment of hESCs gives rise to a CD326–CD56+ population of multipotent mesoderm-committed progenitors (Evseenko et al. 2010). Subsequent, stage-specific modulation of multiple signaling pathways downstream of the early mesendoderm population can recapitulate the human developmental chondrogenic program in human ESCs and iPSCs (Toh et al. 2009; Oldershaw et al. 2010; Wu et al. 2013; Cheng et al. 2014; Craft et al. 2015; Lee et al. 2015). Most early studies employed spontaneous differentiation methods to generate mesenchymal cells, followed by expansion culture using serum-containing MSC media to enrich them. In 2010, Oldershaw and colleagues developed a three-stage, chemically defined directed differentiation protocol which has been developed further by multiple groups for efficient production of differentiated chondrocytes from independent lines of hESCs and iPSCs (Wu et al. 2013; Cheng et al. 2014; Craft et al. 2015; Lee et al. 2015; Oldershaw et al. 2010). In this protocol, human ESCs were directed through a transient primitive streak/mesendoderm stage, followed by controlled differentiation to a multipotent mesoderm and subsequent differentiation of the mesoderm intermediates to chondrogenic cells arranged in aggregates (Oldershaw et al. 2010). By applying temporal supplementation of activin A, bFGF, Wnt3a, BMP4, neurotrophin-4, follistatin, and GDF5, an 8.5-fold expansion of the cell population and 95% expression of the key chondrogenic transcription factor SOX9 are achievable (Oldershaw et al. 2010). Craft and colleagues developed a protocol for chondrogenesis in 3D and provided critical insights into the signaling involved in the controlled specification of hESCs to articular versus hypertrophic chondrocytes (Craft et al. 2015). Moreover, Yamashita and Tsumaki produced scaffoldless cartilage tissue from human iPSCs by use of an extended suspension culture method, although this protocol involved the use of serum (Yamashita et al. 2015).

Developing clinically relevant protocols for directing the differentiation of hESCs into definitive, homogenous populations of chondrogenic cells still faces several challenges. Although hESCs are expandable, it may be efficient to develop methods for the expansion of hPSC-derived chondroprogenitors, which would allow for the generation of chondrocytes from intermediate batches of expandable high-quality controlled cells. Additionally we need to generate tissue in a form optimal for subsequent integration with endogenous cartilage while ensuring quality in the physical properties and long-term durability of the de novo generated cartilage tissue.

6.7 hESC Clinical Trials

The power of hESCs is to generate a platform for the manufacture of potentially all the cell types of the human body. It is the differentiated progeny of hESCs that would be utilized for regenerative medicine and drug screening/toxicity studies, not the

hESCs themselves. Transplanted hESC-derived cells should survive in the recipient without being rejected and differentiate in a site-specific manner, integrate within the target tissue and host circuitry, and restore function. A lot of work is still needed to determine at which stage of differentiation the cells will work best to repair a particular damaged or diseased tissue and how to get those cells to the right place in the body. It is important that the cells are not too immature so avoiding teratoma or more likely progenitor-derived tumor formation, but they should not be too mature either: plastic enough to respond to signals from surrounding cells and host tissue. Currently eight clinical trials using hESC-derived cells are yielding promising results (Ilic and Ogilvie 2017) involving treatment for spinal cord injury, macular degeneration of retina, type 1 diabetes, heart failure, and a hESC-derived dendritic cell for a cancer vaccine. There was a small clinical trial by Geron investigating hESC-derived oligodendrocytes for treatment of spinal cord injury; however, no official results of this trial were published (ClinicalTrials.gov identifier: NCT01217008) and the trial was discontinued for financial reasons. Subsequently, BioTime company Asterias Biotherapeutics acquired all of Geron's stem cell assets and was granted a $14.3 million Strategic Partnership Award by the California Institute for Regenerative Medicine (CIRM) to reinitiate clinical development of hESC-derived OPC1 cells in a dose-escalating trial (ClinicalTrials.gov identifier: NCT02302157). They also acquired the hESC-modified dendritic cell cancer vaccine at the same time.

hESC-derived retinal pigment epithelial (RPE) cells have been tested for treatment of Stargardt's macular dystrophy and dry age-related macular degeneration (AMD) (Schwartz et al. 2012) (ClinicalTrials.gov identifiers: NCT01344993, NCT01345006, NCT01469832), and a phase I/II trial has been approved for testing these cells for the treatment of severe myopia (ClinicalTrials.gov identifier: NCT02122159). Long-term follow-up studies of 15 years for both dry AMD and Stargardt's macular dystrophy trials in the USA are currently ongoing (ClinicalTrials.gov identifiers: NCT02463344 and NCT02445612). A clinical study of a fibrin patch embedded with hESC-derived cardiac-committed progenitors transplanted into patients with severe heart failure commenced in autumn 2014 in France (ClinicalTrials.gov identifier: NCT02057900). Following the treatment, the first patient improved and remained stable 6 months after the intervention (Menasche et al. 2015). The FDA has also approved the ViaCyte phase I clinical trial for the treatment of diabetes using hESC-derived beta cells (ClinicalTrials.gov identifier: NCT02239354) ("Pancreatic Beta Cells").

7 Conclusion

Here we have summarized the progress made to date in the field of human embryonic stem cells from generation, through culture methods and understanding their characteristics to developing strategies to control cell fate. Derivation and culture methods have been significantly improved and simplified, hESC lines can be derived from single biopsied cells of embryos without destroying the embryo, and many directed differentiation protocols are fully chemically defined and have moved away

from using components that would make them unsuitable for human use. Much work still remains to be done in terms of scaling up current protocols to produce sufficient cells for regenerative cell therapies. In addition, more efficient protocols still need to be developed that produce a high proportion of mature desired cells and selection methods to separate these from other lineages in culture without loss of product need to be improved. It is encouraging to note that several clinical trials of human ESC derivatives are under way (Trounson and Dewitt 2016; Ilic and Ogilvie 2017). The medium-term to long-term safety of hESC-derived cells has already been demonstrated in a phase I/II clinical trial (Schwartz et al. 2015) for Stargardt's macular dystrophy and atrophic age-related macular degeneration. However off-target effects still need to be researched. For successful translation, it is important to produce hESC derivatives in a scalable and GMP-compliant manner. Directed differentiation protocols are becoming ever more efficient, and with the increasing availability of GMP reagents, researchers are working toward using fully standardized protocols with xeno-free reagents and defined culture media, carried out under GMP-compliant conditions (Ausubel et al. 2011). Differentiations protocols are now available from many cell types, and hESCs will continue to contribute to the study of human development and disease and to be major tools in the future of regenerative medicine.

Acknowledgments We would like to thank Nicola Bates, Jila Ajeian, Christopher A. Smith, and Alan Kerby for the use of their data in our figures. We thank the UK Medical Research Council (Grants MR/M017344 and MR/L004992/1), the UK Regenerative Medicine Platform hub programme (MR/KO26666/1), BBSRC (BB/D014530/1), and Arthritis Research UK (Grant 20786) for funding the work from our own laboratory.

References

Abeyta MJ, Clark AT, Rodriguez RT, Bodnar MS, Pera RA, Firpo MT (2004) Unique gene expression signatures of independently-derived human embryonic stem cell lines. Hum Mol Genet 13:601–608

Aggarwal VK, Goyal N, Deirmengian G, Rangavajulla A, Parvizi J, Austin MS (2014) Revision total knee arthroplasty in the young patient: is there trouble on the horizon? J Bone Joint Surg Am 96:536–542

Almutawaa W, Rohani L, Rancourt DE (2016) Expansion of human induced pluripotent stem cells in stirred suspension bioreactors. Methods Mol Biol 1502:53–61

Amit M, Itskovitz-Eldor J (2012) Atlas of human pluripotent stem cells: derivation and culturing, New York, Humana Press.

Ang YS, Tsai SY, Lee DF, Monk J, Su J, Ratnakumar K, Ding J, Ge Y, Darr H, Chang B, Wang J, Rendl M, Bernstein E, Schaniel C, Lemischka IR (2011) Wdr5 mediates self-renewal and reprogramming via the embryonic stem cell core transcriptional network. Cell 145:183–197

Ausubel LJ, Lopez PM, Couture LA (2011) GMP scale-up and banking of pluripotent stem cells for cellular therapy applications. Methods Mol Biol 767:147–159

AUTOSTEM (n.d.) Available: http://www.autostem2020.eu/. Accessed 23 Jul 2017

Avior Y, Biancotti JC, Benvenisty N (2015) TeratoScore: assessing the differentiation potential of human pluripotent stem cells by quantitative expression analysis of teratomas. Stem Cell Rep 4:967–974

Badenes SM, Fernandes TG, Cordeiro CS, Boucher S, Kuninger D, Vemuri MC, Diogo MM, Cabral JM (2016) Defined essential 8 medium and vitronectin efficiently support scalable xeno-

free expansion of human induced pluripotent stem cells in stirred microcarrier culture systems. PLoS One 11:e0151264
Barker RA (2014) Developing stem cell therapies for Parkinson's disease: waiting until the time is right. Cell Stem Cell 15:539–542
Barker RA, Barrett J, Mason SL, Bjorklund A (2013) Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. Lancet Neurol 12:84–91
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 9:17–23
Basford CL, Prentice KJ, Hardy AB, Sarangi F, Micallef SJ, Li X, Guo Q, Elefanty AG, Stanley EG, Keller G, Allister EM, Nostro MC, Wheeler MB (2012) The functional and molecular characterisation of human embryonic stem cell-derived insulin-positive cells compared with adult pancreatic beta cells. Diabetologia 55:358–371
Bauwens CL, Peerani R, Niebruegge S, Woodhouse KA, Kumacheva E, Husain M, Zandstra PW (2008) Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. Stem Cells 26:2300–2310
Becker JS, Nicetto D, Zaret KS (2016) H3K9me3-dependent heterochromatin: barrier to cell fate changes. Trends Genet 32:29–41
Beers J, Gulbranson DR, George N, Siniscalchi LI, Jones J, Thomson JA, Chen G (2012) Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. Nat Protoc 7:2029–2040
Bissell DM, Arenson DM, Maher JJ, Roll FJ (1987) Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. J Clin Invest 79:801–812
Bjorklund A, Lindvall O (2000) Cell replacement therapies for central nervous system disorders. Nat Neurosci 3:537–544
Bock C, Kiskinis E, Verstappen G, Gu H, Boulting G, Smith ZD, Ziller M, Croft GF, Amoroso MW, Oakley DH, Gnirke A, Eggan K, Meissner A (2011) Reference maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. Cell 144:439–452
Borger AK, Eicke D, Wolf C, Gras C, Aufderbeck S, Schulze K, Engels L, Eiz-Vesper B, Schambach A, Guzman CA, Lachmann N, Moritz T, Martin U, Blasczyk R, Figueiredo C (2016) Generation of HLA-universal iPSCs-derived megakaryocytes and platelets for survival under refractoriness conditions. Mol Med 22:274–285
Borowiak M, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA (2009) Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell Stem Cell 4:348–358
Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441:349–353
Braam SR, Zeinstra L, Litjens S, Ward-Van Oostwaard D, Van Den Brink S, Van Laake L, Lebrin F, Kats P, Hochstenbach R, Passier R, Sonnenberg A, Mummery CL (2008) Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells 26:2257–2265
Brimble SN, Zeng X, Weiler DA, Luo Y, Liu Y, Lyons IG, Freed WJ, Robins AJ, Rao MS, Schulz TC (2004) Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells Dev 13:585–597
Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa 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
Buecker C, Wysocka J (2012) Enhancers as information integration hubs in development: lessons from genomics. Trends Genet 28:276–284

Buecker C, Chen HH, Polo JM, Daheron L, Bu L, Barakat TS, Okwieka P, Porter A, Gribnau J, Hochedlinger K, Geijsen N (2010) A murine ESC-like state facilitates transgenesis and homologous recombination in human pluripotent stem cells. Cell Stem Cell 6:535–546

Burridge PW, Zambidis ET (2013) Highly efficient directed differentiation of human induced pluripotent stem cells into cardiomyocytes. Methods Mol Biol 997:149–161

Burridge PW, Anderson D, Priddle H, Barbadillo Munoz MD, Chamberlain S, Allegrucci C, Young LE, Denning C (2007) Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. Stem Cells 25:929–938

Buta C, David R, Dressel R, Emgard M, Fuchs C, Gross U, Healy L, Hescheler J, Kolar R, Martin U, Mikkers H, Muller FJ, Schneider RK, Seiler AE, Spielmann H, Weitzer G (2013) Reconsidering pluripotency tests: do we still need teratoma assays? Stem Cell Res 11:552–562

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

Camarasa MV, Galvez VM, Brison DR, Bachiller D (2012) Optimized protocol for derivation of human embryonic stem cell lines. Stem Cell Rev 8:1011–1020

Canham MA, Van Deusen A, Brison DR, De Sousa PA, Downie J, Devito L, Hewitt ZA, Ilic D, Kimber SJ, Moore HD, Murray H, Kunath T (2015) The molecular karyotype of 25 clinical-grade human embryonic stem cell lines. Sci Rep 5:17258

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

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 27:275–280

Chen S, Borowiak M, Fox JL, Maehr R, Osafune K, Davidow L, Lam K, Peng LF, Schreiber SL, Rubin LL, Melton D (2009) A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol 5:258–265

Chen G, Hou Z, Gulbranson DR, Thomson JA (2010) Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. Cell Stem Cell 7:240–248

Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA (2011) Chemically defined conditions for human iPSC derivation and culture. Nat Methods 8:424–429

Chen VC, Couture SM, Ye J, Lin Z, Hua G, Huang HI, Wu J, Hsu D, Carpenter MK, Couture LA (2012) Scalable GMP compliant suspension culture system for human ES cells. Stem Cell Res 8:388–402

Cheng A, Kapacee Z, Peng J, Lu S, Lucas RJ, Hardingham TE, Kimber SJ (2014) Cartilage repair using human embryonic stem cell-derived chondroprogenitors. Stem Cells Transl Med 3:1287–1294

Chin AC, Fong WJ, Goh LT, Philp R, Oh SK, Choo AB (2007) Identification of proteins from feeder conditioned medium that support human embryonic stem cells. J Biotechnol 130:320–328

Chung Y, Klimanskaya I, Becker S, Li T, Maserati M, Lu SJ, Zdravkovic T, Ilic D, Genbacev O, Fisher S, Krtolica A, Lanza R (2008) Human embryonic stem cell lines generated without embryo destruction. Cell Stem Cell 2:113–117

Cohen DE, Melton D (2011) Turning straw into gold: directing cell fate for regenerative medicine. Nat Rev Genet 12:243–252

Craft AM, Rockel JS, Nartiss Y, Kandel RA, Alman BA, Keller GM (2015) Generation of articular chondrocytes from human pluripotent stem cells. Nat Biotechnol 33:638–645

Crocco MC, Fratnz N, Bos-Mikich A (2013) Substrates and supplements for hESCs: a critical review. J Assist Reprod Genet 30:315–323

Curtis A, Riehle M (2001) Tissue engineering: the biophysical background. Phys Med Biol 46: R47–R65

D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534–1541

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, Hyun I, Apperley JF, Barker RA, Benvenisty N, Bredenoord AL, Breuer CK, Caulfield T, Cedars MI, Frey-Vasconcells J, Heslop HE, Jin Y, Lee RT, Mccabe C, Munsie M, Murry CE, Piantadosi S, Rao M, Rooke HM, Sipp D, Studer L, Sugarman J, Takahashi M, Zimmerman M, Kimmelman J (2016) Setting global standards for stem cell research and clinical translation: the 2016 ISSCR guidelines. Stem Cell Rep 6:787–797

Damjanov I, Andrews PW (2016) Teratomas produced from human pluripotent stem cells xenografted into immunodeficient mice – a histopathology atlas. Int J Dev Biol 60:337–419

Datta I, Ganapathy K, Tattikota SM, Bhonde R (2013) Directed differentiation of human embryonic stem cell-line HUES9 to dopaminergic neurons in a serum-free defined culture niche. Cell Biol Int 37:54–64

De los Angeles A, Ferrari F, Xi R, Fujiwara Y, Benvenisty N, Deng H, Hochedlinger K, Jaenisch R, Lee S, Leitch HG, Lensch MW, Lujan E, Pei D, Rossant J, Wernig M, Park PJ, Daley GQ (2015) Hallmarks of pluripotency. Nature 525:469–478

De Peppo GM, Marolt D (2013) Modulating the biochemical and biophysical culture environment to enhance osteogenic differentiation and maturation of human pluripotent stem cell-derived mesenchymal progenitors. Stem Cell Res Ther 4:106

De Sousa PA, Gardner J, Sneddon S, Pells S, Tye BJ, Dand P, Collins DM, Stewart K, Shaw L, Przyborski S, Cooke M, Mclaughlin KJ, Kimber SJ, Lieberman BA, Wilmut I, Brison DR (2009) Clinically failed eggs as a source of normal human embryo stem cells. Stem Cell Res 2:188–197

Ding S, Schultz PG (2004) A role for chemistry in stem cell biology. Nat Biotechnol 22:833–840

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

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

Duggal G, Warrier S, Ghimire S, Broekaert D, Van Der Jeught M, Lierman S, Deroo T, Peelman L, Van Soom A, Cornelissen R, Menten B, Mestdagh P, Vandesompele J, Roost M, Slieker RC, Heijmans BT, Deforce D, De Sutter P, De Sousa Lopes SC, Heindryckx B (2015) Alternative routes to induce naive pluripotency in human embryonic stem cells. Stem Cells 33:2686–2698

Dvash T, Mayshar Y, Darr H, Mcelhaney M, Barker D, Yanuka O, Kotkow KJ, Rubin LL, Benvenisty N, Eiges R (2004) Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. Hum Reprod 19:2875–2883

Efthymiou AG, Chen G, Rao M, Chen G, Boehm M (2014) Self-renewal and cell lineage differentiation strategies in human embryonic stem cells and induced pluripotent stem cells. Expert Opin Biol Ther 14:1333–1344

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

Elliott AM, Elliott KA, Kammesheidt A (2010) High resolution array-CGH characterization of human stem cells using a stem cell focused microarray. Mol Biotechnol 46:234–242

Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689

Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T, Thykjaer T, Maltby E, Smith K, Abu Dawud R, 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

Evseenko D, Zhu Y, Schenke-Layland K, Kuo J, Latour B, Ge S, Scholes J, Dravid G, Li X, Maclellan WR, Crooks GM (2010) Mapping the first stages of mesoderm commitment during differentiation of human embryonic stem cells. Proc Natl Acad Sci U S A 107:13742–13747

Fasano CA, Chambers SM, Lee G, Tomishima MJ, Studer L (2010) Efficient derivation of functional floor plate tissue from human embryonic stem cells. Cell Stem Cell 6:336–347

Figueiredo C, Blasczyk R (2015) A future with less HLA: potential clinical applications of HLA-universal cells. Tissue Antigens 85:443–449

Fong H, Hohenstein KA, Donovan PJ (2008) Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. Stem Cells 26:1931–1938

Frank-Kamenetsky M, Zhang XM, Bottega S, Guicherit O, Wichterle H, Dudek H, Bumcrot D, Wang FY, Jones S, Shulok J, Rubin LL, Porter JA (2002) Small-molecule modulators of hedgehog signaling: identification and characterization of smoothened agonists and antagonists. J Biol 1:10

French A, Bravery C, Smith J, Chandra A, Archibald P, Gold JD, Artzi N, Kim HW, Barker RW, Meissner A, Wu JC, Knowles JC, Williams D, Garcia-Cardena G, Sipp D, Oh S, Loring JF, Rao MS, Reeve B, Wall I, Carr AJ, Bure K, Stacey G, Karp JM, Snyder EY, Brindley DA (2015) Enabling consistency in pluripotent stem cell-derived products for research and development and clinical applications through material standards. Stem Cells Transl Med 4:217–223

Gavrilov S, Marolt D, Douglas NC, Prosser RW, Khalid I, Sauer MV, Landry DW, Vunjak-Novakovic G, Papaioannou VE (2011) Derivation of two new human embryonic stem cell lines from nonviable human embryos. Stem Cells Int 2011:765378

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

Geraghty RJ, Capes-Davis A, Davis JM, Downward J, Freshney RI, Knezevic I, Lovell-Badge R, Masters JR, Meredith J, Stacey GN, Thraves P, Vias M, Cancer Research, U. K (2014) Guidelines for the use of cell lines in biomedical research. Br J Cancer 111:1021–1046

Gertow K, Wolbank S, Rozell B, Sugars R, Andang M, Parish CL, Imreh MP, Wendel M, Ahrlund-Richter L (2004) Organized development from human embryonic stem cells after injection into immunodeficient mice. Stem Cells Dev 13:421–435

Gertow K, Przyborski S, Loring JF, Auerbach JM, Epifano O, Otonkoski T, Damjanov I, Ahrlund-Richter L (2007) Isolation of human embryonic stem cell-derived teratomas for the assessment of pluripotency. Curr Protoc Stem Cell Biol. Chapter 1, Unit1B 4. 3:1B.4.1–1B.4.29

Gertow K, Cedervall J, Jamil S, Ali R, Imreh MP, Gulyas M, Sandstedt B, Ahrlund-Richter L (2011) Early events in xenograft development from the human embryonic stem cell line HS181 – resemblance with an initial multiple epiblast formation. PLoS One 6:e27741

Gifford CA, Ziller MJ, Gu H, Trapnell C, Donaghey J, Tsankov A, Shalek AK, Kelley DR, Shishkin AA, Issner R, Zhang X, Coyne M, Fostel JL, Holmes L, Meldrim J, Guttman M, Epstein C, Park H, Kohlbacher O, Rinn J, Gnirke A, Lander ES, Bernstein BE, Meissner A (2013) Transcriptional and epigenetic dynamics during specification of human embryonic stem cells. Cell 153:1149–1163

Gokhale PJ, Au-Young JK, Dadi S, Keys DN, Harrison NJ, Jones M, Soneji S, Enver T, Sherlock JK, Andrews PW (2015) Culture adaptation alters transcriptional hierarchies among single human embryonic stem cells reflecting altered patterns of differentiation. PLoS One 10: e0123467

Goldring MB, Goldring SR (2007) Osteoarthritis. J Cell Physiol 213:626–634

Gornalusse GG, Hirata RK, Funk SE, Riolobos L, Lopes VS, Manske G, Prunkard D, Colunga AG, Hanafi LA, Clegg DO, Turtle C, Russell DW (2017) HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. Nat Biotechnol 35:765–772

Graham V, Khudyakov J, Ellis P, Pevny L (2003) SOX2 functions to maintain neural progenitor identity. Neuron 39:749–765

Grealish S, Diguet E, Kirkeby A, Mattsson B, Heuer A, Bramoulle Y, Van Camp N, Perrier AL, Hantraye P, Bjorklund A, Parmar M (2014) Human ESC-derived dopamine neurons show

similar preclinical efficacy and potency to fetal neurons when grafted in a rat model of Parkinson's disease. Cell Stem Cell 15:653–665
Grigoriadis AE, Kennedy M, Bozec A, Brunton F, Stenbeck G, Park IH, Wagner EF, Keller GM (2010) Directed differentiation of hematopoietic precursors and functional osteoclasts from human ES and iPS cells. Blood 115:2769–2776
Guo G, Von Meyenn F, Santos F, Chen Y, Reik W, Bertone P, Smith A, Nichols J (2016) Naive pluripotent stem cells derived directly from isolated cells of the human inner cell mass. Stem Cell Rep 6:437–446
Hackett JA, Surani MA (2014) Regulatory principles of pluripotency: from the ground state up. Cell Stem Cell 15:416–430
Harper JC, Pergament E, Delhanty JD (2004) Genetics of gametes and embryos. Eur J Obstet Gynecol Reprod Biol 115(Suppl 1):S80–S84
Healy L, Ruban L (2014) Atlas of human pluripotent stem cells in culture. Springer, New York
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
Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H, Andrews PW (2002) Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20:329–337
Hewitson H, Wood V, Kadeva N, Cornwell G, Codognotto S, Stephenson E, Ilic D (2016) Generation of KCL035 research grade human embryonic stem cell line carrying a mutation in HBB gene. Stem Cell Res 16:210–212
Hikabe O, Hamazaki N, Nagamatsu G, Obata Y, Hirao Y, Hamada N, Shimamoto S, Imamura T, Nakashima K, Saitou M, Hayashi K (2016) Reconstitution in vitro of the entire cycle of the mouse female germ line. Nature 539:299–303
Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development 136:509–523
Houbaviy HB, Murray MF, Sharp PA (2003) Embryonic stem cell-specific MicroRNAs. Dev Cell 5:351–358
Huang SX, Islam MN, O'Neill J, Hu Z, Yang YG, Chen YW, Mumau M, Green MD, Vunjak-Novakovic G, Bhattacharya J, Snoeck HW (2014) Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. Nat Biotechnol 32:84–91
Hunziker EB, Lippuner K, Keel MJ, Shintani N (2015) An educational review of cartilage repair: precepts & practice – myths & misconceptions – progress & prospects. Osteoarthr Cartil 23:334–350
Hwang NS, Varghese S, Elisseeff J (2008) Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration. PLoS One 3:e2498
Hwang Y, Suk S, Lin S, Tierney M, Du B, Seo T, Mitchell A, Sacco A, Varghese S (2013) Directed in vitro myogenesis of human embryonic stem cells and their in vivo engraftment. PLoS One 8: e72023
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 14:122–128
Ilic D, Ogilvie C (2017) Concise review: human embryonic stem cells-what have we done? What are we doing? Where are we going? Stem Cells 35:17–25
International Stem Cell Banking Initiative, C (2009) Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes. Stem Cell Rev 5:301–314
International Stem Cell Banking Initiative, C, 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, Possant 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 25:803–816

International Stem Cell Initiative, C, Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, Christie M, Ford A, Fox V, Gokhale PJ, Healy L, Holm F, Hovatta O, Knowles BB, Ludwig TE, Mckay RD, Miyazaki T, Nakatsuji N, Oh SK, Pera MF, Rossant J, Stacey GN, Suemori H (2010) Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. In Vitro Cell Dev Biol Anim 46:247–258

International Stem Cell Initiative, C, Amps K, Andrews PW, Anyfantis G, Armstrong L, Avery S, Baharvand H, Baker J, Baker D, Munoz MB, Beil S, Benvenisty N, Ben-Yosef D, Biancotti JC, Bosman A, Brena RM, Brison D, Caisander G, Camarasa MV, Chen J, Chiao E, Choi YM, Choo AB, Collins D, Colman A, Crook JM, Daley GQ, Dalton A, De Sousa PA, Denning C, Downie J, Dvorak P, Montgomery KD, Feki A, Ford A, Fox V, Fraga AM, Frumkin T, Ge L, Gokhale PJ, Golan-Lev T, Gourabi H, Gropp M, Lu G, Hampl A, Harron K, Healy L, Herath W, Holm F, Hovatta O, Hyllner J, Inamdar MS, Irwanto AK, Ishii T, Jaconi M, Jin Y, Kimber S, Kiselev S, Knowles BB, Kopper O, Kukharenko V, Kuliev A, Lagarkova MA, Laird PW, Lako M, Laslett AL, Lavon N, Lee DR, Lee JE, Li C, Lim LS, Ludwig TE, Ma Y, Maltby E, Mateizel I, Mayshar Y, Mileikovsky M, Minger SL, Miyazaki T, Moon SY, Moore H, Mummery C, Nagy A, Nakatsuji N, Narwani K, Oh SK, Oh SK, Olson C, Otonkoski T, Pan F, Park IH, Pells S, Pera MF, Pereira LV, Qi O, Raj GS, Reubinoff B, Robins A, Robson P, Rossant J et al (2011) Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. Nat Biotechnol 29:1132–1144

Inzunza J, Gertow K, Stromberg MA, Matilainen E, Blennow E, Skottman H, Wolbank S, Ahrlund-Richter L, Hovatta O (2005) Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23:544–549

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

Joannides AJ, Fiore-Heriche C, Battersby AA, Athauda-Arachchi P, Bouhon IA, Williams L, Westmore K, Kemp PJ, Compston A, Allen ND, Chandran S (2007) A scaleable and defined system for generating neural stem cells from human embryonic stem cells. Stem Cells 25:731–737

Jones CA, Pohar S (2012) Health-related quality of life after total joint arthroplasty: a scoping review. Clin Geriatr Med 28:395–429

Kadzik RS, Morrisey EE (2012) Directing lung endoderm differentiation in pluripotent stem cells. Cell Stem Cell 10:355–361

Kalia LV, Lang AE (2015) Parkinson's disease. Lancet 386:896–912

Karwacki-Neisius V, Goke J, Osorno R, Halbritter F, Ng JH, Weisse AY, Wong FC, Gagliardi A, Mullin NP, Festuccia N, Colby D, Tomlinson SR, Ng HH, Chambers I (2013) Reduced Oct4 expression directs a robust pluripotent state with distinct signaling activity and increased enhancer occupancy by Oct4 and Nanog. Cell Stem Cell 12:531–545

Kashyap V, Rezende NC, Scotland KB, Shaffer SM, Persson JL, Gudas LJ, Mongan NP (2009) Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. Stem Cells Dev 18:1093–1108

Katsetos CD, Legido A, Perentes E, Mork SJ (2003) Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. J Child Neurol 18:851–866. discussion 867

Kaur J, Tilkins ML (2013) Methods for culturing human embryonic stem cells on feeders. Methods Mol Biol 997:93–113

Kearns NA, Genga RM, Ziller M, Kapinas K, Peters H, Brehm MA, Meissner A, Maehr R (2013) Generation of organized anterior foregut epithelia from pluripotent stem cells using small molecules. Stem Cell Res 11:1003–1012

Kefalopoulou Z, Politis M, Piccini P, Mencacci N, Bhatia K, Jahanshahi M, Widner H, Rehncrona S, Brundin P, Bjorklund A, Lindvall O, Limousin P, Quinn N, Foltynie T (2014) Long-term clinical outcome of fetal cell transplantation for Parkinson disease: two case reports. JAMA Neurol 71:83–87

Kehoe DE, Jing D, Lock LT, Tzanakakis ES (2010) Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. Tissue Eng Part A 16:405–421

Kerz M, Folarin A, Meleckyte R, Watt FM, Dobson RJ, Danovi D (2016) A novel automated high-content analysis workflow capturing cell population dynamics from induced pluripotent stem cell live imaging data. J Biomol Screen 21:887–896

Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-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 DS, Kim JY, Kang M, Cho MS, Kim DW (2007) Derivation of functional dopamine neurons from embryonic stem cells. Cell Transplant 16:117–123

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 467:285–290

King FW, Liszewski W, Ritner C, Bernstein HS (2011) High-throughput tracking of pluripotent human embryonic stem cells with dual fluorescence resonance energy transfer molecular beacons. Stem Cells Dev 20:475–484

Kirkeby A, Grealish S, Wolf DA, Nelander J, Wood J, Lundblad M, Lindvall O, Parmar M (2012) Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. Cell Rep 1:703–714

Kleinman HK, Mcgarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21:6188–6193

Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R (2006) Human embryonic stem cell lines derived from single blastomeres. Nature 444:481–485

Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R (2007) Derivation of human embryonic stem cells from single blastomeres. Nat Protoc 2:1963–1972

Koay EJ, Hoben GM, Athanasiou KA (2007) Tissue engineering with chondrogenically differentiated human embryonic stem cells. Stem Cells 25:2183–2190

Kopp JL, Ormsbee BD, Desler M, Rizzino A (2008) Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells 26:903–911

Krawetz RJ, Li X, Rancourt DE (2009) Human embryonic stem cells: caught between a ROCK inhibitor and a hard place. BioEssays 31:336–343

Krawetz R, Taiani JT, Liu S, Meng G, Li X, Kallos MS, Rancourt DE (2010) Large-scale expansion of pluripotent human embryonic stem cells in stirred-suspension bioreactors. Tissue Eng Part C Methods 16:573–582

Kriks 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

Krishnakumar R, Blelloch RH (2013) Epigenetics of cellular reprogramming. Curr Opin Genet Dev 23:548–555

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

Kshitiz PJ, Kim P, Helen W, Engler AJ, Levchenko A, Kim DH (2012) Control of stem cell fate and function by engineering physical microenvironments. Integr Biol (Camb) 4:1008–1018
Kurosawa H (2007) Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. J Biosci Bioeng 103:389–398
Kurpinski K, Chu J, Hashi C, LI S (2006) Anisotropic mechanosensing by mesenchymal stem cells. Proc Natl Acad Sci U S A 103:16095–16100
Kwon GS, Fraser ST, Eakin GS, Mangano M, Isern J, Sahr KE, Hadjantonakis AK, Baron MH (2006) Tg(Afp-GFP) expression marks primitive and definitive endoderm lineages during mouse development. Dev Dyn 235:2549–2558
Lakshmipathy U, Love B, Goff LA, Jornsten R, Graichen R, Hart RP, Chesnut JD (2007) MicroRNA expression pattern of undifferentiated and differentiated human embryonic stem cells. Stem Cells Dev 16:1003–1016
Lamba DA, Karl MO, Ware CB, Reh TA (2006) Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci U S A 103:12769–12774
Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA (2013) Cerebral organoids model human brain development and microcephaly. Nature 501:373–379
Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, Martin W, Fornwald J, Lehr R, Harling J, Gaster L, Callahan JF, Olson BA (2002) Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol 62:58–64
Laurent LC, Ulitsky I, Slavin I, Tran H, Schork A, Morey R, Lynch C, Harness JV, Lee S, Barrero MJ, Ku S, Martynova M, Semechkin R, Galat V, Gottesfeld J, Izpisua Belmonte JC, Murry C, Keirstead HS, Park HS, Schmidt U, Laslett AL, Muller FJ, Nievergelt CM, Shamir R, Loring JF (2011) Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. Cell Stem Cell 8:106–118
Lee ST, Oh SW, Kim DY, Han JY, Moon SY, Lim JM (2006) Serum replacement with a growth factor-free synthetic substance in culture medium contributes to effective establishment of mouse embryonic stem cells of various origins. Fertil Steril 86:1137–1145
Lee J, Taylor SE, Smeriglio P, Lai J, Maloney WJ, Yang F, Bhutani N (2015) Early induction of a prechondrogenic population allows efficient generation of stable chondrocytes from human induced pluripotent stem cells. FASEB J 29:3399–3410
Leitch HG, Mcewen KR, Turp A, Encheva V, Carroll T, Grabole N, Mansfield W, Nashun B, Knezovich JG, Smith A, Surani MA, Hajkova P (2013) Naive pluripotency is associated with global DNA hypomethylation. Nat Struct Mol Biol 20:311–316
Lerou PH, Yabuuchi A, Huo H, Takeuchi A, Shea J, Cimini T, Ince TA, Ginsburg E, Racowsky C, Daley GQ (2008) Human embryonic stem cell derivation from poor-quality embryos. Nat Biotechnol 26:212–214
Li M, Izpisua Belmonte JC (2016) Looking to the future following 10 years of induced pluripotent stem cell technologies. Nat Protoc 11:1579–1585
Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP (2013) Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. Nat Protoc 8:162–175
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 2:1187–1203
Lim CY, Solter D, Knowles BB, Damjanov I (2015) Development of teratocarcinomas and teratomas in severely immunodeficient NOD.Cg-Prkdc(scid) Il2rg(tm1Wjl)/Szj (NSG) mice. Stem Cells Dev 24:1515–1520
Lin Y, Chen G (2014) Embryoid body formation from human pluripotent stem cells in chemically defined E8 media (June 1, 2014). In: Stembook E (ed) The stem cell research community, StemBook
Lindvall O, Bjorklund A (2004) Cell therapy in Parkinson's disease. NeuroRx 1:382–393
Lindvall O, Kokaia Z (2009) Prospects of stem cell therapy for replacing dopamine neurons in Parkinson's disease. Trends Pharmacol Sci 30:260–267

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462:315–322

Liu Y, Liu H, Sauvey C, Yao L, Zarnowska ED, Zhang SC (2013) Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. Nat Protoc 8:1670–1679

Loh KM, Chen A, Koh PW, Deng TZ, Sinha R, Tsai JM, Barkal AA, Shen KY, Jain R, Morganti RM, Shyh-Chang N, Fernhoff NB, George BM, Wernig G, Salomon RE, Chen Z, Vogel H, Epstein JA, Kundaje A, Talbot WS, Beachy PA, Ang LT, Weissman IL (2016) Mapping the pairwise choices leading from pluripotency to human bone, heart, and other mesoderm cell types. Cell 166:451–467

Ludwig TE, Bergendahl V, Levenstein ME, Yu J, Probasco MD, Thomson JA (2006a) Feeder-independent culture of human embryonic stem cells. Nat Methods 3:637–646

Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS, Llanas RA, Thomson JA (2006b) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24:185–187

Lukovic D, Diez Lloret A, Stojkovic P, Rodriguez-Martinez D, Perez Arago MA, Rodriguez-Jimenez FJ, Gonzalez-Rodriguez P, Lopez-Barneo J, Sykova E, Jendelova P, Kostic J, Moreno-Manzano V, Stojkovic M, Bhattacharya SS, Erceg S (2017) Highly efficient neural conversion of human pluripotent stem cells in adherent and animal-free conditions. Stem Cells Transl Med 6:1217–1226

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 1:55–70

Maroof AM, Keros S, Tyson JA, Ying SW, Ganat YM, Merkle FT, Liu B, Goulburn A, Stanley EG, Elefanty AG, Widmer HR, Eggan K, Goldstein PA, Anderson SA, Studer L (2013) Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. Cell Stem Cell 12:559–572

Marti M, Mulero L, Pardo C, Morera C, Carrio M, Laricchia-Robbio L, Esteban CR, Izpisua Belmonte JC (2013) Characterization of pluripotent stem cells. Nat Protoc 8:223–253

Martin MJ, Muotri A, Gage F, Varki A (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 11:228–232

Martinat C, Bacci JJ, Leete T, Kim J, Vanti WB, Newman AH, Cha JH, Gether U, Wang H, Abeliovich A (2006) Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A 103:2874–2879

Mascetti VL, Pedersen RA (2016) Human-mouse chimerism validates human stem cell pluripotency. Cell Stem Cell 18:67–72

Masters JR, Stacey GN (2007) Changing medium and passaging cell lines. Nat Protoc 2:2276–2284

Mccracken KW, Howell JC, Wells JM, Spence JR (2011) Generating human intestinal tissue from pluripotent stem cells in vitro. Nat Protoc 6:1920–1928

Mckay TR, Camarasa MV, Iskender B, Ye J, Bates N, Miller D, Fitzsimmons JC, Foxler D, Mee M, Sharp TV, Aplin J, Brison DR, Kimber SJ (2011) Human feeder cell line for derivation and culture of hESc/hiPSc. Stem Cell Res 7:154–162

Mehta A, Mathew S, Viswanathan C, Sen Majumdar A (2010) Intrinsic properties and external factors determine the differentiation bias of human embryonic stem cell lines. Cell Biol Int 34:1021–1031

Menasche P, Vanneaux V, Hagege A, Bel A, Cholley B, Cacciapuoti I, Parouchev A, Benhamouda N, Tachdjian G, Tosca L, Trouvin JH, Fabreguettes JR, Bellamy V, Guillemain R, Suberbielle Boissel C, Tartour E, Desnos M, Larghero J (2015) Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report. Eur Heart J 36:2011–2017

Meng G, Liu S, Rancourt DE (2012) Synergistic effect of medium, matrix, and exogenous factors on the adhesion and growth of human pluripotent stem cells under defined, xeno-free conditions. Stem Cells Dev 21:2036–2048

Mikkola M, Olsson C, Palgi J, Ustinov J, Palomaki T, Horelli-Kuitunen N, Knuutila S, Lundin K, Otonkoski T, Tuuri T (2006) Distinct differentiation characteristics of individual human embryonic stem cell lines. BMC Dev Biol 6:40

Miyazono M, Lee VM, Trojanowski JQ (1995) Proliferation, cell death, and neuronal differentiation in transplanted human embryonal carcinoma (NTera2) cells depend on the graft site in nude and severe combined immunodeficient mice. Lab Investig 73:273–283

Moustakas A, Heldin CH (2009) The regulation of TGFbeta signal transduction. Development 136:3699–3714

Muller FJ, Brandl B, Loring JF (2008) Assessment of human pluripotent stem cells with PluriTest. StemBook, Cambridge, MA

Muller FJ, Goldmann J, Loser P, Loring JF (2010) A call to standardize teratoma assays used to define human pluripotent cell lines. Cell Stem Cell 6:412–414

Muller FJ, Schuldt BM, Williams R, Mason D, Altun G, Papapetrou EP, Danner S, Goldmann JE, Herbst A, Schmidt NO, Aldenhoff JB, Laurent LC, Loring JF (2011) A bioinformatic assay for pluripotency in human cells. Nat Methods 8:315–317

Murdoch A, Braude P, Courtney A, Brison D, Hunt C, Lawford-Davies J, Moore H, Stacey G, Sethe S, Procurement Working Group Of National Clinical H, E. S. C. F, National Clinical H, E. S. C. F (2012) The procurement of cells for the derivation of human embryonic stem cell lines for therapeutic use: recommendations for good practice. Stem Cell Rev 8:91–99

Na J, Baker D, Zhang J, Andrews PW, Barbaric I (2014) Aneuploidy in pluripotent stem cells and implications for cancerous transformation. Protein Cell 5:569–579

Nakazawa K, Yoshiura Y, Koga H, Sakai Y (2013) Characterization of mouse embryoid bodies cultured on microwell chips with different well sizes. J Biosci Bioeng 116:628–633

Ng ES, Davis RP, Hatzistavrou T, Stanley EG, Elefanty AG (2008) Directed differentiation of human embryonic stem cells as spin embryoid bodies and a description of the hematopoietic blast colony forming assay. Curr Protoc Stem Cell Biol, Chapter 1, Unit 1D 3. 4:1D.3.1-1D.3.23

Nicholas CR, Chen J, Tang Y, Southwell DG, Chalmers N, Vogt D, Arnold CM, Chen YJ, Stanley EG, Elefanty AG, Sasai Y, Alvarez-Buylla A, Rubenstein JL, Kriegstein AR (2013) Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. Cell Stem Cell 12:573–586

Nostro MC, Sarangi F, Ogawa S, Holtzinger A, Corneo B, Li X, Micallef SJ, Park IH, Basford C, Wheeler MB, Daley GQ, Elefanty AG, Stanley EG, Keller G (2011) Stage-specific signaling through TGFbeta family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Development 138:861–871

Nukaya D, Minami K, Hoshikawa R, Yokoi N, Seino S (2015) Preferential gene expression and epigenetic memory of induced pluripotent stem cells derived from mouse pancreas. Genes Cells 20:367–381

Ohi Y, Qin H, Hong C, Blouin L, Polo JM, Guo T, Qi Z, Downey SL, Manos PD, Rossi DJ, Yu J, Hebrok M, Hochedlinger K, Costello JF, Song JS, Ramalho-Santos M (2011) Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. Nat Cell Biol 13:541–549

Oldershaw RA, Baxter MA, Lowe ET, Bates N, Grady LM, Soncin F, Brison DR, Hardingham TE, Kimber SJ (2010) Directed differentiation of human embryonic stem cells toward chondrocytes. Nat Biotechnol 28:1187–1194

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 122:3169–3179

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

Pagliuca FW, Melton DA (2013) How to make a functional beta-cell. Development 140:2472–2483

Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH, Peterson QP, Greiner D, Melton DA (2014) Generation of functional human pancreatic beta cells in vitro. Cell 159:428–439

Patterson M, Chan DN, Ha I, Case D, Cui Y, Van Handel B, Mikkola HK, Lowry WE (2012) Defining the nature of human pluripotent stem cell progeny. Cell Res 22:178–193

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci U S A 101:12543–12548

Perestrelo T, Chen W, Correia M, Le C, Pereira S, Rodrigues AS, Sousa MI, Ramalhosantos J, Wirtz D (2017) Pluri-IQ: quantification of embryonic stem cell pluripotency through an image-based analysis software. Stem Cell Rep 9:697–709

Pesl M, Acimovic I, Pribyl J, Hezova R, Vilotic A, Fauconnier J, Vrbsky J, Kruzliak P, Skladal P, Kara T, Rotrekl V, Lacampagne A, Dvorak P, Meli AC (2014) Forced aggregation and defined factors allow highly uniform-sized embryoid bodies and functional cardiomyocytes from human embryonic and induced pluripotent stem cells. Heart Vessel 29:834–846

Peterson S, Loring JF (2012) Human stem cell manual: a laboratory guide. Academic Press

Phillips BW, Hentze H, Rust WL, Chen QP, Chipperfield H, Tan EK, Abraham S, Sadasivam A, Soong PL, Wang ST, Lim R, Sun W, Colman A, Dunn NR (2007) Directed differentiation of human embryonic stem cells into the pancreatic endocrine lineage. Stem Cells Dev 16:561–578

Pijuan-Galito S, Tamm C, Schuster J, Sobol M, Forsberg L, Merry CL, Anneren C (2016) Human serum-derived protein removes the need for coating in defined human pluripotent stem cell culture. Nat Commun 7:12170

Pistollato F, Bremer-Hoffmann S, Healy L, Young L, Stacey G (2012) Standardization of pluripotent stem cell cultures for toxicity testing. Expert Opin Drug Metab Toxicol 8:239–257

Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, Apostolou E, Stadtfeld M, Li Y, Shioda T, Natesan S, Wagers AJ, Melnick A, Evans T, Hochedlinger K (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat Biotechnol 28:848–855

Przyborski SA (2005) Differentiation of human embryonic stem cells after transplantation in immune-deficient mice. Stem Cells 23:1242–1250

Ramirez JM, Gerbal-Chaloin S, Milhavet O, Qiang B, Becker F, Assou S, Lemaitre JM, Hamamah S, De Vos J (2011) Brief report: benchmarking human pluripotent stem cell markers during differentiation into the three germ layers unveils a striking heterogeneity: all markers are not equal. Stem Cells 29:1469–1474

Rao TP, Kuhl M (2010) An updated overview on Wnt signaling pathways: a prelude for more. Circ Res 106:1798–1806

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399–404

Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J, Gauvin R, Narayan K, Karanu F, O'Neil JJ, Ao Z, Warnock GL, Kieffer TJ (2012) Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 61:2016–2029

Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, O'dwyer S, Quiskamp N, Mojibian M, Albrecht T, Yang YH, Johnson JD, Kieffer TJ (2014) Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 32:1121–1133

Richards M, Tan SP, Tan JH, Chan WK, Bongso A (2004) The transcriptome profile of human embryonic stem cells as defined by SAGE. Stem Cells 22:51–64

Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K (2010) Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol 28:611–615

Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, Domogatskaya A, Xiao Z, Damdimopoulou P, Sheikhi M, Inzunza J, Nilsson AS, Baker D, Kuiper R, Sun Y, Blennow E, Nordenskjold M, Grinnemo KH, Kere J, Betsholtz C, Hovatta O, Tryggvason K (2014) Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. Nat Commun 5:3195

Roelandt P, Pauwelyn KA, Sancho-Bru P, Subramanian K, Bose B, Ordovas L, Vanuytsel K, Geraerts M, Firpo M, De Vos R, Fevery J, Nevens F, Hu WS, Verfaillie CM (2010) Human

embryonic and rat adult stem cells with primitive endoderm-like phenotype can be fated to definitive endoderm, and finally hepatocyte-like cells. PLoS One 5:e12101

Roost MS, Van Iperen L, Ariyurek Y, Buermans HP, Arindrarto W, Devalla HD, Passier R, Mummery CL, Carlotti F, De Koning EJ, Van Zwet EW, Goeman JJ, Chuva De Sousa Lopes SM (2015) KeyGenes, a tool to probe tissue differentiation using a human fetal transcriptional atlas. Stem Cell Rep 4:1112–1124

Rosowski KA, Mertz AF, Norcross S, Dufresne ER, Horsley V (2015) Edges of human embryonic stem cell colonies display distinct mechanical properties and differentiation potential. Sci Rep 5:14218

Rubin LL (2008) Stem cells and drug discovery: the beginning of a new era? Cell 132:549–552

Santangelo P, Nitin N, Bao G (2006) Nanostructured probes for RNA detection in living cells. Ann Biomed Eng 34:39–50

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

Schiffer CA (2001) Diagnosis and management of refractoriness to platelet transfusion. Blood Rev 15:175–180

Schulz TC (2015) Concise review: manufacturing of pancreatic endoderm cells for clinical trials in type 1 diabetes. Stem Cells Transl Med 4:927–931

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 379:713–720

Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, Hubschman JP, Davis JL, Heilwell G, Spirn M, Maguire J, Gay R, Bateman J, Ostrick RM, Morris D, Vincent M, Anglade E, Del Priore LV, Lanza R (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 385:509–516

Seltmann S, Lekschas F, Muller R, Stachelscheid H, Bittner MS, Zhang W, Kidane L, Seriola A, Veiga A, Stacey G, Kurtz A (2016) hPSCreg – the human pluripotent stem cell registry. Nucleic Acids Res 44:D757–D763

Serra M, Brito C, Sousa MF, Jensen J, Tostoes R, Clemente J, Strehl R, Hyllner J, Carrondo MJ, Alves PM (2010) Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control. J Biotechnol 148:208–215

Shi Y, Kirwan P, Livesey FJ (2012) Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. Nat Protoc 7:1836–1846

Shirane K, Kurimoto K, Yabuta Y, Yamaji M, Satoh J, Ito S, Watanabe A, Hayashi K, Saitou M, Sasaki H (2016) Global landscape and regulatory principles of DNA methylation reprogramming for germ cell specification by mouse pluripotent stem cells. Dev Cell 39:87–103

Sinagoga KL, Wells JM (2015) Generating human intestinal tissues from pluripotent stem cells to study development and disease. EMBO J 34:1149–1163

Singh U, Quintanilla RH, Grecian S, Gee KR, Rao MS, Lakshmipathy U (2012) Novel live alkaline phosphatase substrate for identification of pluripotent stem cells. Stem Cell Rev 8:1021–1029

Skalli O, Pelte MF, Peclet MC, Gabbiani G, Gugliotta P, Bussolati G, Ravazzola M, Orci L (1989) Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. J Histochem Cytochem 37:315–321

Smith JR, Vallier L, Lupo G, Alexander M, Harris WA, Pedersen RA (2008) Inhibition of activin/nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. Dev Biol 313:107–117

Solomon S, Pitossi F, Rao MS (2015) Banking on iPSC – is it doable and is it worthwhile. Stem Cell Rev 11:1–10

Solter D, Knowles BB (1975) Immunosurgery of mouse blastocyst. Proc Natl Acad Sci U S A 72:5099–5102

Soteriou D, Iskender B, Byron A, Humphries JD, Borg-Bartolo S, Haddock MC, Baxter MA, Knight D, Humphries MJ, Kimber SJ (2013) Comparative proteomic analysis of supportive and

unsupportive extracellular matrix substrates for human embryonic stem cell maintenance. J Biol Chem 288:18716–18731
Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko VV, Wells SI, Zorn AM, Shroyer NF, Wells JM (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470:105–109
Sperger JM, Chen X, Draper JS, Antosiewicz JE, Chon CH, Jones SB, Brooks JD, Andrews PW, Brown PO, Thomson JA (2003) Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Natl Acad Sci U S A 100:13350–13355
Stacey GN (2011) Cell culture contamination. Methods Mol Biol 731:79–91
Stacey G (2012) Banking stem cells for research and clinical applications. Prog Brain Res 200:41–58
Stacey G, Hunt CJ (2006) The UK stem cell bank: a UK government-funded, international resource center for stem cell research. Regen Med 1:139–142
Steiner D, Khaner H, Cohen M, Even-Ram S, Gil Y, Itsykson P, Turetsky T, Idelson M, Aizenman E, Ram R, Berman-Zaken Y, Reubinoff B (2010) Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension. Nat Biotechnol 28:361–364
Steward MM, Lee JS, O'Donovan A, Wyatt M, Bernstein BE, Shilatifard A (2006) Molecular regulation of H3K4 trimethylation by ASH2L, a shared subunit of MLL complexes. Nat Struct Mol Biol 13:852–854
Strom S, Inzunza J, Grinnemo KH, Holmberg K, Matilainen E, Stromberg AM, Blennow E, Hovatta O (2007) Mechanical isolation of the inner cell mass is effective in derivation of new human embryonic stem cell lines. Hum Reprod 22:3051–3058
Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 62:3603–3608
Suo G, Han J, Wang X, Zhang J, Zhao Y, Zhao Y, Dai J (2005) Oct4 pseudogenes are transcribed in cancers. Biochem Biophys Res Commun 337:1047–1051
Swistowski A, Peng J, Han Y, Swistowska AM, Rao MS, Zeng X (2009) Xeno-free defined conditions for culture of human embryonic stem cells, neural stem cells and dopaminergic neurons derived from them. PLoS One 4:e6233
Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Kang E, Fulati A, Lee HS, Sritanaudomchai H, Masterson K, Larson J, Eaton D, Sadler-Fredd K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer RL, Wolf D, Mitalipov S (2013) Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 153:1228–1238
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takasato M, Er PX, Becroft M, Vanslambrouck JM, Stanley EG, Elefanty AG, Little MH (2014) Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. Nat Cell Biol 16:118–126
Takeuchi H, Nakatsuji N, Suemori H (2014) Endodermal differentiation of human pluripotent stem cells to insulin-producing cells in 3D culture. Sci Rep 4:4488
Tannenbaum SE, Turetsky TT, Singer O, Aizenman E, Kirshberg S, Ilouz N, Gil Y, Berman-Zaken Y, Perlman TS, Geva N, Levy O, Arbell D, Simon A, Ben-Meir A, Shufaro Y, Laufer N, Reubinoff BE (2012) Derivation of xeno-free and GMP-grade human embryonic stem cells – platforms for future clinical applications. PLoS One 7:e35325
TAPBIOSYSTEMS (n.d.) Available: http://www.tapbiosystems.com/tap/cell_culture/CompacT_SelecT.htm. Accessed 14-10-2016
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
Taylor CJ, Peacock S, Chaudhry AN, Bradley JA, Bolton EM (2012) Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types. Cell Stem Cell 11:147–152

TECAN (n.d.) Available: http://ww3.tecan.com/platform/content/element/5549/Tecan%20Cellerity_Package.pdf. Accessed 14-10-2016

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

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

Tieng V, Stoppini L, Villy S, Fathi M, Dubois-Dauphin M, Krause KH (2014) Engineering of midbrain organoids containing long-lived dopaminergic neurons. Stem Cells Dev 23:1535–1547

Toh WS, Yang Z, Heng BC, Cao T (2007) Differentiation of human embryonic stem cells toward the chondrogenic lineage. Salamanders Regen Res Methods Protocol 407:333–349

Toh WS, Guo XM, Choo AB, Lu K, Lee EH, Cao T (2009) Differentiation and enrichment of expandable chondrogenic cells from human embryonic stem cells in vitro. J Cell Mol Med 13:3570–3590

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 51:1754–1765

Trounson A, Dewitt ND (2016) Pluripotent stem cells progressing to the clinic. Nat Rev Mol Cell Biol 17:194–200

Tsankov AM, Akopian V, Pop R, Chetty S, Gifford CA, Daheron L, Tsankova NM, Meissner A (2015) A qPCR ScoreCard quantifies the differentiation potential of human pluripotent stem cells. Nat Biotechnol 33:1182–1192

Turetsky T, Aizenman E, Gil Y, Weinberg N, Shufaro Y, Revel A, Laufer N, Simon A, Abeliovich D, Reubinoff BE (2008) Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. Hum Reprod 23:46–53

Turner N, Grose R (2010) Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer 10:116–129

Vallier L, Reynolds D, Pedersen RA (2004) Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev Biol 275:403–421

Van Hoof D, Liku ME (2013) Directed differentiation of human pluripotent stem cells along the pancreatic endocrine lineage. Methods Mol Biol 997:127–140

Vaskova EA, Stekleneva AE, Medvedev SP, Zakian SM (2013) "Epigenetic memory" phenomenon in induced pluripotent stem cells. Acta Nat 5:15–21

Vegas AJ, Veiseh O, Gurtler M, Millman JR, Pagliuca FW, Bader AR, Doloff JC, Li J, Chen M, Olejnik K, Tam HH, Jhunjhunwala S, Langan E, Aresta-Dasilva S, Gandham S, Mcgarrigle JJ, Bochenek MA, Hollister-Lock J, Oberholzer J, Greiner DL, Weir GC, Melton DA, Langer R, Anderson DG (2016) Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 22:306–311

Vendrame F, Pileggi A, Laughlin E, Allende G, Martin-Pagola A, Molano RD, Diamantopoulos S, Standifer N, Geubtner K, Falk BA, Ichii H, Takahashi H, Snowhite I, Chen Z, Mendez A, Chen L, Sageshima J, Ruiz P, Ciancio G, Ricordi C, Reijonen H, Nepom GT, Burke GW 3rd, Pugliese A (2010) Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells. Diabetes 59:947–957

Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R (1999) Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 156:71–83

Vestergaard ML, Awan A, Warzecha CB, Christensen ST, Andersen CY (2016) Immunofluorescence microscopy and mRNA analysis of human embryonic stem cells (hESCs) including primary cilia associated signaling pathways. Methods Mol Biol 1307:123–140

Vitillo L, Baxter M, Iskender B, Whiting P, Kimber SJ (2016) Integrin-associated focal adhesion kinase protects human embryonic stem cells from apoptosis, detachment, and differentiation. Stem Cell Rep 7:167–176

Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, Watanabe TK, Tanigami A (2003) Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology (Oxford) 42:162–165

Wang X, Lin G, Martins-Taylor K, Zeng H, Xu RH (2009) Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells. J Biol Chem 284:34054–34064

Wang Y, Chou BK, Dowey S, He C, Gerecht S, Cheng L (2013) Scalable expansion of human induced pluripotent stem cells in the defined xeno-free E8 medium under adherent and suspension culture conditions. Stem Cell Res 11:1103–1116

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K, Sasai Y (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686

Weathersbee PS, Pool TB, Ord T (1995) Synthetic serum substitute (SSS): a globulin-enriched protein supplement for human embryo culture. J Assist Reprod Genet 12:354–360

Weinberger L, Ayyash M, Novershtern N, Hanna JH (2016) Dynamic stem cell states: naive to primed pluripotency in rodents and humans. Nat Rev Mol Cell Biol 17:155–169

Weissbein U, Benvenisty N, Ben-David U (2014) Quality control: genome maintenance in pluripotent stem cells. J Cell Biol 204:153–163

Whiting P, Kerby J, Coffey P, Da Cruz L, Mckernan R (2015) Progressing a human embryonic stem-cell-based regenerative medicine therapy towards the clinic. Philos Trans R Soc Lond Ser B Biol Sci 370:20140375

Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385–397

Widuchowski W, Widuchowski J, Trzaska T (2007) Articular cartilage defects: study of 25,124 knee arthroscopies. Knee 14:177–182

Williams RC, Opelz G, Mcgarvey CJ, Weil EJ, Chakkera HA (2016) The risk of transplant failure with HLA mismatch in first adult kidney allografts from deceased donors. Transplantation 100:1094–1102

Wolf DP, Morey R, Kang E, Ma H, Hayama T, Laurent LC, Mitalipov S (2016) Embryonic stem cells derived by somatic cell nuclear transfer: a horse in the race? Stem Cells 35(1):26–34

Wu L, Bluguermann C, Kyupelyan L, Latour B, Gonzalez S, Shah S, Galic Z, Ge S, Zhu Y, Petrigliano FA, Nsair A, Miriuka SG, Li X, Lyons KM, Crooks GM, Mcallister DR, Van Handel B, Adams JS, Evseenko D (2013) Human developmental chondrogenesis as a basis for engineering chondrocytes from pluripotent stem cells. Stem Cell Rep 1:575–589

Xia Y, Nivet E, Sancho-Martinez I, Gallegos T, Suzuki K, Okamura D, Wu MZ, Dubova I, Esteban CR, Montserrat N, Campistol JM, Izpisua Belmonte JC (2013) Directed differentiation of human pluripotent cells to ureteric bud kidney progenitor-like cells. Nat Cell Biol 15:1507–1515

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971–974

Yamashita A, Morioka M, Yahara Y, Okada M, Kobayashi T, Kuriyama S, Matsuda S, Tsumaki N (2015) Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs. Stem Cell Rep 4:404–418

Yan Y, Yang D, Zarnowska ED, Du Z, Werbel B, Valliere C, Pearce RA, Thomson JA, Zhang SC (2005) Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. Stem Cells 23:781–790

Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM (2008) Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453:524–528

Ye J, Bates N, Soteriou D, Grady L, Edmond C, Ross A, Kerby A, Lewis PA, Adeniyi T, Wright R, Poulton KV, Lowe M, Kimber SJ, Brison DR (2017) High quality clinical grade human embryonic stem cell lines derived from fresh discarded embryos. Stem Cell Res Ther 8:128

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

Young L, Sung J, Stacey G, Masters JR (2010) Detection of mycoplasma in cell cultures. Nat Protoc 5:929–934

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 318:1917–1920

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

Use of Xenogeneic Cells

Elisabeth Kemter, Alexander Kind, Bruno Reichart, and Eckhard Wolf

Contents

E. Kemter
Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences; and Center for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Munich, Germany
e-mail: kemter@genzentrum.lmu.de

A. Kind
Chair for Livestock Biotechnology, TUM School of Life Sciences Weihenstephan, Technische Universität München, Freising, Germany
e-mail: kind@wzw.tum.de

B. Reichart
Walter-Brendel-Center of Experimental Medicine, Ludwig-Maximilians-Universität München, Munich, Germany
e-mail: Bruno.Reichart@med.uni-muenchen.de

E. Wolf (✉)
Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences; and Center for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Munich, Germany

German Center for Diabetes Research (DZD), Neuherberg, Germany
e-mail: ewolf@genzentrum.lmu.de

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_23

Abstract

Xenotransplantation of cells, tissues, and organs is a rapidly developing field. The pig is the favorite donor species for a number of reasons, including similarity with humans in the size and physiology of many organs, high fecundity, and the possibility of genetic modification. This chapter provides an overview of the immunological and physiological hurdles facing pig-to-primate cell and tissue xenotransplantation and strategies to overcome them, with particular emphasis on porcine pancreatic islets, as these have already been used clinically. We also describe the current state of the art in xenotransplantation of porcine cornea, neuronal cells, skin, hepatocytes, chondrocytes, red blood cells, Sertoli cells, and mesenchymal stem cells. Major progress has been made, particularly in the development of novel immunosuppressive regimens and genetically multimodified donor pigs. Xenotransplantation of porcine cells and tissues has the potential to become a clinically relevant option for the treatment of degenerative diseases and traumatic tissue defects. Ethical and regulatory frameworks for this new branch of medicine are currently being developed.

1 Introduction

Regenerative therapies involving auto- or allotransplantation of cells or tissues are already a clinical reality, e.g., skin transplantation in patients with major skin loss (Zhou et al. 2013), or islet transplantation to cure diabetes mellitus (Dunn et al. 2015). Autologous transplantation is the graft of cells or tissues from one part of the body to another in the same individual, whereas allografts are derived from another individual of the same species. Human autologous transplantation is restricted by the amount of tissue available, and allogeneic procedures are limited by the availability of suitable donated cells and tissues, which invariably fall short of the clinical need. The use of stem cell-derived cell and tissue grafts is one means of overcoming these problems, as discussed elsewhere in this book. Another promising new option is xenotransplantation, that is, the use of animals as a source of replacement cells and tissues for human therapy (Perkel 2016).

The pig is currently favored as the donor species of xenografts for human patients for a number of reasons (Ekser et al. 2015a), including

(i) Anatomical and physiological similarities to humans
(ii) High fecundity and abundant donor supply, allowing selection of high-quality grafts
(iii) Efficient and precise techniques for genetic modification
(iv) Low risk of pathogen transmission through housing under designated pathogen-free (DPF) conditions and continuous health monitoring
(v) Less socio-cultural and ethical concerns compared to research involving non-human primates (NHPs) or dogs

However, clinical use of xenogeneic cells faces considerable hurdles, such as activation of the recipient's immune system by xenogeneic antigens, and functional incompatibilities or deficits of the porcine graft.

Major advances have been made in recent years, and clinical realization of porcine cell, tissue, and even organ transplants now seems possible. Results of a clinical study of microencapsulated pig islets have been reported (Matsumoto et al. 2016; Cooper et al. 2016a); and neuronal cell and acellular corneal stroma xenotransplantation studies are imminent (Ekser et al. 2012a).

This chapter provides an overview of the status of pig-to-primate cell and tissue transplantation, the biological hurdles they face and the strategies to overcome them. We do not cover xenotransplantation of vascularized organs or decellularized tissue-derived scaffolds. For recent reviews of these topics, the interested reader is referred to Cooper et al. (2014), Mohiuddin et al. (2015), Iwase and Kobayashi (2015), Ekser et al. (2015b), Laird et al. (2016) and to the book "Biomaterials" in this book series on "Tissue Engineering and Regeneration."

2 Definition of Xenogeneic Cell-Based Therapy

The World Health Organization (WHO) defines xenotransplantation products as "*living cells, tissues or organs of animal origin, and human body fluids, cells, tissues or organs that have ex vivo contact with these living, xenogeneic materials: and that has the potential to constitute an alternative to material of human origin and bridge the shortfall in human material for transplantation*" (WHO).

The European Medicines Agency (EMA) defines xenogeneic cell-based therapy and xenogeneic cell-based medicinal products in guideline EMEA/CHMP/CPWP/83508/2009 (EMA 2009):

> **Xenogeneic cell-based therapy** is the use of viable animal somatic cell preparations, suitably adapted for: (a) implantation/infusion into a human recipient or (b) extracorporeal treatment through bringing (non-human) animal cells into contact with human body fluids, tissues or organs. The principal objective is reconstitution of cell/tissue/organ functions. The genotype and/or phenotype of the cells may have been modified, e.g., by isolation, culture, expansion, pharmacological treatment or combination with various matrices.
>
> **Xenogeneic cell-based medicinal products** contain viable animal cells or tissues as the active substance. Xenogeneic materials might be sourced either from non-transgenic or transgenic animals. The animal cells can also be genetically modified.

3 Immunological Obstacles to Pig-to-Primate Xenotransplantation

The immunological obstacles to successful cell and tissue xenotransplantation have been studied most extensively for pancreatic islets, but are broadly relevant for other cell and tissue xenografts. Xenogeneic islet cell engraftment and survival is curtailed for several reasons (Fig. 1) (Klymiuk et al. 2016; Park et al. 2015):

(i) Cell death during isolation, culture and after transplantation
(ii) Impaired oxygen supply due to slow or poor neo-vascularization
(iii) Early islet loss after intraportal transplantation due to an instant blood mediated inflammatory reaction (IBMIR)
(iv) Humoral and cellular transplant rejection, activation of the (partly dissimilar) recipient's coagulation system, activation of the recipient's complement system

Most of these problems are also encountered during allogeneic transplantation, but IBMIR and other graft rejection mechanisms are more severe in xenotransplantation.

The following section draws mainly from the pig islet xenotransplantation literature and describes different mechanisms of xenograft loss and, in Sect. 5, various strategies to improve engraftment and survival. These mechanisms are – at least in part – also relevant for other cell and tissue xenografts.

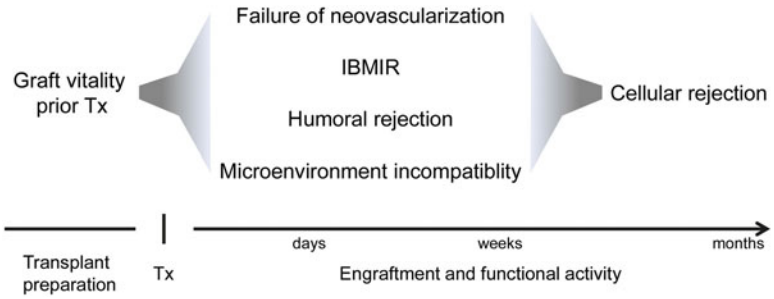


Fig. 1 The biological outcome of cell and tissue grafts. Cell and tissue allo- and xenografts are subject to primary nonfunction and cellular rejection. Primary nonfunction is the immediate failure of the newly implanted cells or tissues to engraft and the graft never shows biological function. It can be caused by various factors, like failure of neovascularization, graft rejection by IBMIR and/or antibody-mediated graft loss, and microenvironment incompatibility (e.g., poor engraftment of hepatocytes in the microenvironment of a cirrhotic liver). Tx: transplantation; IBMIR: instant blood mediated inflammatory reaction

3.1 Instant Blood Mediated Inflammatory Reaction (IBMIR)

Intraportal infusion into the liver, the route of clinical auto- and allotransplantation of islets using the Edmonton immunosuppression protocol (Shapiro et al. 2006), triggers IBMIR, an innate immune response that occurs within a few hours (van der Windt et al. 2007). IBMIR is associated with activation of complement and coagulation, endothelial activation, cytokine and chemokine release, inflammatory cell activation, infiltration of the graft, platelet aggregation on the islet surface, and thrombus formation (reviewed in Kourtzelis et al. 2015). These thrombo-inflammatory reactions can result in immediate loss of up to 70% of the infused islet mass (van der Windt et al. 2007). A very large number of islets are thus required to achieve a functional graft.

IBMIR occurs not only after islet transplantation, but also after intraportal infusion of other cell types such as hepatocytes (Gustafson et al. 2011), or mesenchymal stem cells (Moll et al. 2011). The mechanisms of IBMIR are generally similar between allogeneic and xenogeneic cell transplantation, but the relevance of individual components may differ (reviewed in Nilsson et al. 2011). Antibodies and complement play a more prominent role in xeno- than in allogeneic islet transplantation. This is due to the presence of preformed antibodies in the recipient against specific xenoantigens, such as galactosyl-α1,3-galactose (αGal) synthesized by α-1,3-galactosyltransferase (GGTA1), N-acetylneuraminic acid (Neu5Gc) synthesized by cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), and an Sd(a)-like glycan made by porcine β-1,4-N-acetyl-galactosaminyl transferase 2 (B4GALNT2) (reviewed in Byrne et al. 2015). Binding of these preformed antibodies to their xenoantigens leads to activation of the complement system, which cannot be controlled due to species incompatibilities between regulators on the xenograft and the recipient's effector molecules (reviewed in Kourtzelis

et al. 2015). Furthermore, there are molecular incompatibilities of the coagulation homeostasis system between the porcine tissue and the human recipient. For example, tissue factor (TF), which is expressed and secreted by porcine pancreatic alpha- and beta-cells, activates the coagulation cascade (van der Windt et al. 2007). Controversial results have been obtained in different experimental settings regarding whether porcine TF pathway inhibitor (TFPI) can prevent activation of the human coagulation cascade (reviewed in Cowan and Robson 2015). A recent study demonstrated that Kunitz domain 1 is key to the incompatibility between porcine TFPI and human TF and that clotting can be inhibited by expressing human TFPI in porcine cells (Ji et al. 2015).

3.2 Humoral Immune Response to Cell and Tissue Xenotransplants

Humoral graft rejection is triggered by antibodies directed against the xenograft. These antibodies may be preformed or produced de novo by B cells after recognition of xenoepitopes (reviewed in Vadori and Cozzi 2015). Recognition of "nonself" xenogeneic epitopes and subsequent B-cell activation can be triggered by T cells and natural killer (NK) cells. In general, antibodies are less important in the rejection of cell and tissue xenografts, which become vascularized by the recipient vessel system, than in vascularized organs where the binding of antibodies to the porcine vascular endothelium initiates complement-induced endothelial injury and graft rejection (reviewed in Yang and Sykes 2007).

As mentioned above, αGal is the most significant porcine cell surface epitope against which humans have preformed antibodies (reviewed in Vadori and Cozzi 2015). αGal is widely expressed in nonprimate mammals and synthesized by the enzyme GGTA1. This enzyme is defective in humans and old-world monkeys, which consequently lack αGal. Immunogenic contact with bacterial αGal epitopes in the intestinal tract stimulates production of anti-αGal antibodies in early postnatal life. In nonprimate mammals, αGal abundance varies between different cell types and tissues, and age-related differences in αGal abundance have been observed. αGal epitopes are present at high levels in porcine fetal and neonatal islet-like cell clusters (NICCs), but are almost absent in adult pig islets (Rayat et al. 2003). The benefit of blocking or removing αGal epitopes in pig-to-primate islet xenotransplantation experiments thus depends on the source used (reviewed in Klymiuk et al. 2016).

Approximately 13% IgM and 36% IgG in sera of healthy humans bind to non-αGal epitopes on porcine endothelial cells (Baumann et al. 2007). One of these is Neu5Gc (Hanganutziu-Deicher antigen) synthesized by CMAH, an enzyme expressed in mammals including pigs and higher primates, but not birds or humans (Morozumi et al. 1999). Neu5Gc has been detected on various porcine cell types including endothelial cells, as well as adult and fetal pig islets.

Recent comparison of N-glycan moieties of pig and human islets has revealed further non-αGal and non-Neu5Gc immunogenic epitopes on porcine cells

(Miyagawa et al. 2014). Adult pig islets express several high-mannose type N-glycans not detected in human islets, including an Sd(a)-like glycan made by the enzyme B4GALNT2 (reviewed in Byrne et al. 2015).

Besides immunogenic cell surface sugar moieties, porcine cells and tissues are also recognized as "nonself" by the recipient's immune system and induce humoral immune responses, as are allografts (reviewed in Vadori and Cozzi 2015). Antibody-mediated graft rejection is exacerbated if patients are presensitized and their serum contains antibodies against donor major histocompatibility complex (MHC) class I molecules/human leukocyte antigens (HLAs). Anti-HLA-antibodies cross-react with swine MHC subclasses/swine leukocyte antigens (SLAs), since specific MHC epitopes are conserved between the two species (Mulder et al. 2010). This cross-reactivity might be detrimental for long-term xenograft survival. In addition, human T-cell receptors can bind porcine SLA complexes, triggering human T-cell activation (Hara et al. 2013), with different SLA polymorphisms eliciting strong or weak stimulatory effects. Xenograft survival can therefore be supported by avoiding donor pigs with strongly stimulating SLA alleles (Lunney et al. 2009).

3.3 Cellular Immune Response to Cell and Tissue Transplants

Both innate and adaptive components of the cellular immune system contribute to xenograft rejection (reviewed in Griesemer et al. 2014). Cells of the innate immune system comprise neutrophils, NK cells, and monocytes/macrophages, while B and T lymphocytes are part of the adaptive immune system. The immune response is controlled by tight regulation of immune cell activity by activating and inhibitory signals. In xenotransplantation, molecular incompatibilities between receptors and inhibitory ligands from different species can negatively affect graft success.

Immune cell infiltration of tissue and solid organ xenografts starts with neutrophils as an early responding cell population, followed by macrophages and T cells (reviewed in Vadori and Cozzi 2015). Neutrophils are able to distinguish between xenogeneic and allogeneic cells. Notably, neutrophil attraction and attack of xenogeneic cells is independent of αGal epitopes and can occur in the absence of complement (Al-Mohanna et al. 2005). Monocytes and differentiated macrophages play important roles in the cellular response against xenografts (reviewed in Vadori and Cozzi 2015). Macrophage activation can occur in a T-cell dependent and independent manner. $CD4^+$ T-cell-mediated macrophage activation has been identified as a principal mechanism of islet xenograft destruction. But T-cell independent activation of macrophages can also destroy porcine islet xenografts, even when microencapsulated. Encapsulated islets secrete chemotactic factors, including macrophage inflammatory protein-1 alpha (MIP-1α alias C-C motif chemokine ligand 3, CCL3) that attract macrophages and myofibroblasts, resulting in pericapsular fibrosis, impaired access to nutrients, and islet death. In addition to their phagocytic activity, macrophages modulate the adaptive immunity of both $CD4^+$ and $CD8^+$ T cells by contributing to cell recruitment and antigen presentation. The phagocytic activity of macrophages is tightly regulated by receptor-ligand interactions, e.g., by

the interaction of the inhibitory "don't eat me" signal regulatory protein α (SIRPα) receptor on monocytes/macrophages with the ubiquitously expressed CD47 molecule, preventing phagocytosis of autologous cells. SIRPα on human phagocytic cells cannot recognize porcine CD47 on xenogeneic cells, potentially leading to their destruction (Navarro-Alvarez and Yang 2011, 2014).

Immune-cell-mediated rejection of xenografts such as porcine islets is primarily a $CD4^+$ T-cell dependent process, possibly triggered by stimulation of macrophage effector activity ((Karlsson-Parra et al. 1996); reviewed in Vadori and Cozzi 2015). T cells can also be activated by direct binding to porcine SLA class 1 and class 2 molecules, or indirectly by recipient antigen-presenting cells (APCs) expressing MHCs with processed xenoantigens (reviewed in Vadori and Cozzi 2015). Indirect T-cell activation triggered by xenografts might be stronger than by allografts, because of the large number of xenogeneic peptides presented by human APCs. Peptides derived from SLA class 1 molecules, widely expressed on porcine cells, are considered as particularly immunogenic. Activated T cells can in turn mediate several other cellular anti-xenograft responses, such as activation of NK and B cells and of innate immune mechanisms.

Two signals are usually required for T-cell activation. One is T-cell receptor (TCR) signaling, which encompasses binding of the TCR to an MHC-peptide complex on an APC. The other is a co-stimulatory signal, which may – depending on its nature – induce and amplify an effective immune response, or have an inhibitory tolerogenic function. T-cell activation is regulated by numerous co-stimulatory pathways (Fig. 2). In xenotransplantation, the best studied T-cell co-stimulatory signaling complexes are CD80/CD86-CD28 and CD40-CD154, with CD28 and CD154 (=CD40L) being localized on T cells and CD80/CD86 and CD40 on APCs. Blocking these pathways by drugs or expression of immunomodulators in genetically engineered donor pigs are important strategies to overcome T-cell mediated rejection. These are discussed in Sect. 5.

4 Further Hurdles to Achieve Long-Term Function of Xenogeneic Cell and Tissue Grafts

4.1 Failure of Neo-Vascularization

Sufficient oxygen and nutrient supply is essential for cell survival and function, and pancreatic islets are especially sensitive (reviewed in (Wang et al. 2011)). During islet isolation and culture, the endogenous capillary network is destroyed. After transplantation, cell and tissue grafts (e.g., islets, cornea, and skin) derive their blood supply through in-growth of blood vessels from the recipient. However, re-vascularization takes time (approximately 7–14 days), and the regenerated vascular network of transplanted islets differs from the original and is often functionally insufficient (Wang et al. 2011). Promoting revascularization and preventing beta-cell apoptosis, especially during hypoxia, can thus significantly improve islet graft function and survival. Various means of stimulating revascularization are discussed in Sect. 5.5.

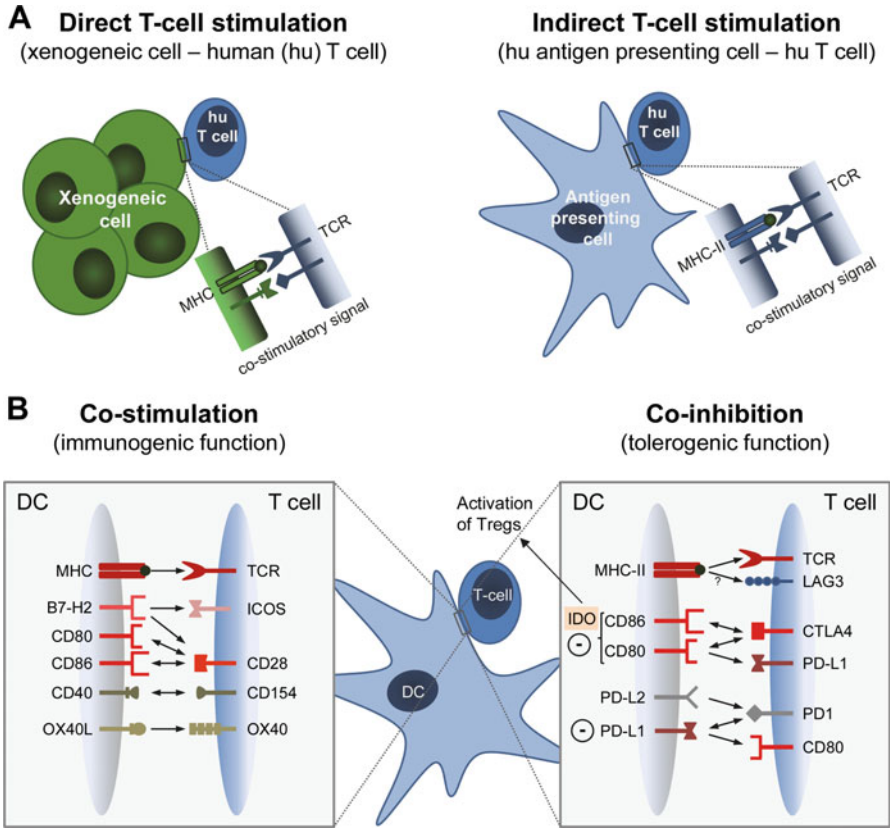


Fig. 2 Mechanisms of T-cell co-stimulation and regulation of T-cell stimulation and inhibition by co-signaling interactions. (**a**) Activation of recipient's T cells can occur directly by binding of peptides loaded MHC class I or class II of xenogeneic cells (antigen-presenting cell or any cell expressing MHC). For indirect xenorecognition to occur, xenogeneic proteins must be first processed by recipient's antigen presenting cells like dendritic cells (DCs). Subsequently, peptides derived from these xenogeneic antigens are cross-presented by recipients MHC class II on recipient's antigen presenting cell. Comparing the intensity of T-cell activation between allo- and xenotransplantation, the direct xenogeneic and allogeneic T-cell responses appear to be equivalent, but the indirect xenogeneic response seems to be stronger. (**b**) Besides T-cell receptor (TCR) signaling, full activation of T cells requires co-stimulatory signal. Depending on kind of co-stimulatory receptor-ligand interaction, an immunogenic or tolerogenic T-cell immune response can be the outcome. The figure shows receptors and ligands of indirect T-cell stimulation and co-stimulation by antigen-presenting DCs. (Adapted from Chen and Flies (2013) and from Bakdash et al. (2013))

4.2 Physiological/Functional Differences of Cells and Tissues between Pig and Human

A recent study revealed important differences in glucose-stimulated insulin secretion (GSIS) in vitro between porcine and human islets (Mueller et al. 2013). The GSIS of

islets from adult and young (< 3 months) pigs was only around 30% and 3% of human islets. The insulin content of adult pig islets is similar to that of human islets, whereas that of juvenile pig islets is 80% less. Thus, significantly more porcine islets than human islets may be required to achieve a fully functional graft. The minimum number of nonencapsulated pig islets transplanted to achieve normoglycemia in diabetic NHPs was 25,000 islet equivalents (IEQ; clusters of >150 μm in diameter)/kg body weight or greater for adult islets, and twofold higher for neonatal islet-like cell clusters, though much larger numbers have been transplanted in some studies (reviewed in Bartlett et al. 2016). Human recipients should require fewer porcine IEQ per kg body weight, because insulin requirements per kg are two- to threefold lower than in streptozotocin (STZ)-diabetic NHPs. Nevertheless, it is reasonable to estimate that islets from three or more adult pig pancreata will be required to achieve insulin independence for one type 1 diabetic patient (Bartlett et al. 2016). Genetic modifications to improve the insulin secretion capacity of porcine islets have recently been proposed (see Sect. 5.7).

5 Strategies to Overcome Immune Rejection and Functional/Physiological Incompatibilities of Cell and Tissue Xenografts

As summarized in Sect. 3, prolonged survival of cell and tissue xenografts may be hampered by activation of the recipient's coagulation, complement, and/or immune systems. The nature and intensity of the transplant injury and the ultimate outcome are influenced by the cells or tissues transplanted (whether vascularized, whether xenoreactive epitopes are present), the transplant site, and the invasiveness of the transplantation procedure. Strategies to improve long-term survival include:

(i) Improvement of therapeutic interventions, e.g., systemic immunosuppression with reduced side effects, strengthening of local immunoregulation
(ii) In vitro modification of transplantable cells and tissues, e.g., by encapsulation
(iii) Optimization of the cell or tissue isolation and transplantation process, e.g., by selecting the most suitable transplant site
(iv) Genetic modification of the donor animal to provide an optimized transplant (Cooper et al. 2016b)

5.1 Systemic Immunosuppression vs. Local Immune Modulation

Standard systemic immunosuppressive regimes, e.g., combinations of tacrolimus, cyclosporine, mycophenolate mofetil, or rapamycin, do not prevent xenograft rejection unless used in high doses that inevitably risk complications such as infection (reviewed in Cooper et al. 2016c). The use of T-cell co-stimulation blocking agents does however offer encouraging improvements and these drugs are highly effective in selectively blocking T-cell activation and preventing an elicited antibody

response. The initial agent used to block the CD40-CD154 T-cell co-stimulation pathway, anti-CD154 monoclonal antibody (mAb), was found to be thrombogenic in humans (Kawai et al. 2000) and has been replaced by an anti-CD40 mAb with similar activity. Blockade of the CD80/CD86-CD28 co-stimulation pathway can be achieved by systemic treatment with CTLA4-Ig (abatacept®), or a modified version with higher affinity for CD86 (LEA29Y = belatacept®). Alternatively, donor pigs can be genetically engineered to express T-cell co-stimulation blocking molecules (see Sect. 5.4.4). Another possibility is to provide local immune protection of the xenograft by co-transplanting cells that secrete immunomodulators. Examples include testicular Sertoli cells (see Sect. 6.8) and mesenchymal stem cells (see Sect. 6.9).

5.2 Protection of Xenogeneic Cells by Encapsulation

Immune isolation of cells by encapsulation is a promising strategy for both allo- and xenogeneic cell transplantation. This avoids lifelong immunosuppression and should be readily implemented in the clinic (reviewed in Bartlett et al. 2016). Permselective membranes protect the transplanted cells against the recipient's immune system, but allow oxygen and nutrient supply (reviewed in Mahou et al. 2016). Immune isolation approaches can be classified as intravascular devices directly connected to the recipient's circulatory system by vascular anastomosis and extravascular devices. Extravascular devices include macroencapsulation and microencapsulation strategies where cells are enclosed in 150 to 1,000 μm hydrogel polymer spheres (reviewed in Mahou et al. 2016; Dufrane and Gianello 2012). These prevent access of immune cells and antibodies, while oxygen, nutrients, insulin, and other hormones can pass through. Lower molecular weight chemokines and cytokines may also reach the graft, but their deleterious effects are reduced by the increased physical distance between encapsulated cells and chemo-/cytokine-producing cells of the innate immune system (reviewed in Barkai et al. 2016).

A detailed overview of encapsulated islet transplantation studies in rodents and large animals was published recently by Robles et al. (2014).

5.2.1 Microencapsulation

Microencapsulation has been applied to xenogeneic pancreatic islets (Elliott et al. 2005, 2007; reviewed in Cooper et al. 2016a), and hepatocytes (Ham et al. 2015; reviewed in Meier et al. 2015). Microcapsules may contain one or a few islets (reviewed in Yang and Yoon 2015), or a few hundred hepatocytes (Jitraruch et al. 2014). A general finding is that peri-capsular fibrosis induced by immune responses against the capsulating material limits survival of encapsulated cells due to sub-optimal oxygenation. Further progress requires optimization of the material and structure of permselective membranes, oxygen supply, diffusion properties, size of the encapsulation device, and the preparation and homogenous encapsulation of the cells. This is in itself a field in biomaterial research (reviewed in Bartlett et al. 2016).

The interested reader is referred to recent review articles (Barkai et al. 2016; Ludwig and Ludwig 2015).

Progress continues in long-term immune isolation strategies. The alginate biomaterials initially used for cell encapsulation elicited strong innate immune responses resulting in pericapsular fibrosis (Elliott et al. 2005; de Groot et al. 2004). The use of chemically imidazole-modified alginate, such as triazole-thiomorpholine dioxide (TMTD) alginate, or chitosan-coated alginate mitigates foreign-body responses and has prolonged microencapsulated graft function in animal models (Vegas et al. 2016a, b; Yang et al. 2015a). Nonalginate-based biomaterials, such as poly(ethylene) glycol (PEG), or new low immunogenic polymers (e.g., new glucose polymers) are also under investigation (reviewed in Bartlett et al. 2016).

Since microcapsules with islets are too large for intraportal transplantation and have diffusional limitations, conformal coating of porcine islets using a thin layer of photopolymerizable polyethylene glycol has been investigated (reviewed in Klymiuk et al. 2016). This approach has recently been refined to achieve high-throughput encapsulation of individual mouse islets into nanoliter droplets of optimized PEG gel, resulting in a thin (tens of micrometers) continuous layer of hydrogel. Conformal coating did not impair the ability of the islets to restore normoglycemia in syngeneic diabetic mice (Tomei et al. 2014). However, no efficacy studies of conformal coated xenoislets in diabetic large animal models have yet been published (reviewed in Klymiuk et al. 2016).

5.2.2 Macroencapsulation

Macroencapsulation devices contain the total transplanted cell mass within a single, confined device, which is commonly used in the extravascular mode (reviewed in Bartlett et al. 2016; Yang and Yoon 2015). They can be easily transplanted and also retrieved or reloaded with new cells and have greater mechanical strength than microcapsules. Several studies have demonstrated that macroencapsulated porcine islets could restore glucose homeostasis in diabetic NHP models (reviewed in Klymiuk et al. 2016). However, the primary challenge of this approach is inefficient nutrient access and product release, especially when the device is avascular in the immediate postoperative course.

Pierre Gianello's group (Catholic University of Louvain, Belgium) reported a small trial with diabetic NHPs treated with subcutaneously implanted macrodevices (alginate patches incorporating porcine islets) that controlled hyperglycemia for up to 6 months (Dufrane et al. 2010). Although no fibrosis or complement deposition was observed on explanted macrodevices, IgG anti-pig antibodies, mainly against the αGal epitope, were found in the recipients. A subsequent study demonstrated that co-encapsulation of pig islets with mesenchymal stem cells improved implant oxygenation and neoangiogenesis, but the long-term function of a subcutaneous bioartificial pancreas in NHPs was only slightly improved (Veriter et al. 2014).

The company Beta-O2 Technologies (Rosh-Haayin, Israel) have developed a macroencapsulation device in which pancreatic islets are embedded in a multilayer immune-protective membrane of alginates and a polytetrafluoroethylene (PTFE)

membrane that prevents access of immunoglobulins while allowing sufficient oxygen from a central chamber (Neufeld et al. 2013). Ambient air is provided via two subcutaneous ports, and this enables adequate supply and survival of islets in large retrievable macrocapsules implanted into poorly oxygenated sites like the subcutis. The concept of islet macroencapsulation was successfully demonstrated in different transplant models in large animals (biocompatibility, efficacy, immune-protection) (Ludwig et al. 2012) and then in a trial using allogeneic islets in a human patient (Ludwig et al. 2013). This latter study showed persistent islet graft function in the chamber for 10 months with regulated insulin secretion and preserved islet morphology without immunosuppression. A study of porcine islet xenotransplantation in diabetic NHPs was subsequently initiated, and the first promising results reported at the IPITA-IXA-CTS Conference 2015 in Melbourne (Ludwig et al. 2015) and published recently (Ludwig et al. 2017).

Other macroencapsulation devices tested with human islets or stem cell-derived cell types have been reviewed recently (Yang and Yoon 2015), some of which may be relevant for xenogeneic cells in future (Zhu et al. 2015).

5.3 Optimization of the Isolation Procedure and Selection of the Transplantation Site

The survival of cell and tissue allo- and xenografts is acutely influenced by the quality and quantity of the transplants, the implantation site, and the surgical technique used (reviewed in Bartlett et al. 2016). Success of both allo- and xenotransplantation of pancreatic islets critically depends on optimized and standardized pancreas procurement and islet isolation protocols. While human pancreata originate mainly from deceased donors located some distance from the recipient, procurement of porcine material can be coordinated locally, resulting in short ischemia times and optimal conditions for islet isolation and transplantation. Quality control of islets encompasses islet yield, purity, viability, and function (as tested by static glucose stimulated insulin secretion), as well as microbiological safety tests and screening for endotoxins (reviewed in Qi 2014; Shapiro 2012). For porcine neonatal islet-like cell clusters (NICCs), which are immature at the time of isolation, in vitro or in vivo maturation is required before they can restore normoglycemia in diabetic recipients (Jimenez-Vera et al. 2015). Culture and in vitro maturation of NICCs for 12 days has been shown to be better than shorter periods. Pretransplant "islet preconditioning" to improve clinical outcome is also a major research focus in allotransplantation. This includes coating of islets with compounds that improve oxygenation and promote islet engraftment, protect against hypoxic stress, reduce IBMIR, provide local immunosuppression, or enable in vivo islet imaging (reviewed in Bartlett et al. 2016). In islet xenotransplantation, these preconditioning strategies can also be complemented by genetically modifying the donor pigs (see Sects. 5.4 and 5.5).

While intraportal infusion into the liver is the usual route for clinical islet allo- and autotransplantation (Shapiro et al. 2006), this incurs the risk of IBMIR, even more so with xenogeneic islets. Alternative transplantation sites have therefore been tested

where islets are not immediately exposed to blood, including skeletal muscles, peritoneum and omental pouches, bone marrow, and subcutis (reviewed in Bartlett et al. 2016; Fotino et al. 2015).

5.4 Immunoprotection and Improved Survival of Xenografts from Genetically Engineered Donor Animals

Genetic engineering of the donor pigs has increased the survival of pig-to-primate xenografts from minutes to months and, most recently, years (Mohiuddin et al. 2016; reviewed in Cooper et al. 2016c). With the availability of highly efficient tools for genetic engineering, notably the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) system, in combination with somatic cell nuclear transfer (reviewed in Klymiuk et al. 2015), the number of genetically engineered pig lines is rapidly increasing. At the time of writing, more than 40 genetic alterations have been introduced into pigs to improve their suitability as xenotransplant donors (Cooper et al. 2016c). Recommendations on genetic modifications of donor pigs for islet xenotransplantation have been published recently by the International Xenotransplantation Association (IXA) (Cowan et al. 2016). These genetic modifications include removal of major xenoantigens, rectifying species incompatibilities in complement and coagulation regulation, and expression of molecules capable of local immunomodulation or supporting graft survival. Genetic modifications that reduce the risk of infections have also been proposed (reviewed in Denner and Mueller 2015). It is likely that the most suitable combination of genetic alterations will differ according to the intended xenotransplantation procedure, e.g., depending on

(i) The type of cell or tissue
(ii) The transplantation site
(iii) The nature of additional immunoprotection measures, e.g., encapsulation

The generation of clinically useful donor pigs will require the addition of many transgenes, so the recent development of "combineering" and gene stacking to combine multiple transgenes at a single chromosomal site, avoiding segregation during breeding of the donor animals, is a useful advance (Fischer et al. 2016).

5.4.1 Elimination of Major Xenoantigens

Hyperacute rejection is initiated by preformed antibodies against αGal epitopes, resulting in complement activation and rapid graft destruction. It can be overcome by genetic inactivation of the α-1,3-galactosyltransferase (*GGTA1*) gene. Following the first publication on GGTA1 deficient pigs (Phelps et al. 2003), multiple *GGTA1* knockout pig lines were generated, initially by gene targeting (reviewed in Klymiuk et al. 2010) and later by gene editing (e.g., Hauschild et al. 2011). Genetic elimination of αGal epitopes was a major step towards realization of vascularized organ xenotransplantation (reviewed in Vadori and Cozzi 2015; Klymiuk et al. 2010).

Although the αGal epitope is less important for cell and tissue transplants than vascularized organs (porcine endothelial cells have high levels of αGal epitopes), removing αGal epitopes improves the long-term outcome of porcine skin, islet, and – to a lesser extent – cornea xenografts (see Sect. 6). In porcine cornea, the major xenogeneic antigens are αGal and, more prominently, Neu5Gc (Cohen et al. 2014). Pigs lacking CMAH have been generated to remove Neu5Gc (Kwon et al. 2013) and combined with GGTA1 deficiency (Lutz et al. 2013). Pigs lacking a further major xenoantigen, an Sd(a)-like glycan, have been produced by inactivating the *B4GALNT2* gene (Estrada et al. 2015). The authors showed that cells from GGTA1/CMAH/B4GALNT2 deficient pigs exhibited reduced human IgM and IgG binding compared to cells lacking only GGTA1 and CMAH.

5.4.2 Diminishing Pro-inflammatory Signals

Metabolic and mechanical stress associated with islet isolation and culture can lead to activation of the NF-κB and mitogen-activated kinase (MAPK) stress response pathways and release of inflammatory mediators, including monocyte chemotactic protein 1/chemokine (C-C motif) ligand 2 (MCP1/CCL2), interleukin 1 beta (IL1B), tumor necrosis factor alpha (TNFA), interleukin 6 (IL6), and nitric oxide (NO). These may trigger or exacerbate thrombotic and inflammatory responses to allo- or xenoislets, not only after intraportal transplantation but also at alternative transplantation sites (reviewed in Wilson and Chaikof 2008; Martin et al. 2015; Cowley et al. 2012). In addition, tissue factor (TF), the primary physiological activator of the coagulation system and a known important player in IBMIR (reviewed in Kourtzelis et al. 2015), is released by islets after isolation. Surface expression of TF on islet cells is stimulated by CCL2 in an auto- and paracrine manner, further increasing the pro-inflammatory state of the islets (Moberg et al. 2002). Blocking of CCL2 improved long-term survival of murine islet allografts (Lee et al. 2003). Inactivation of the *CCL2* gene and transgenic overexpression of tissue factor pathway inhibitor (TFPI) were recently proposed as a means of reducing IBMIR by diminishing pro-inflammatory and pro-coagulant signals from islet xenografts (reviewed in Klymiuk et al. 2016; Bartlett et al. 2016). Human (h) TFPI transgenic pig islets (on a *GGTA1*-KO/hCD46 transgenic background) were shown to mitigate IBMIR and reduce early cell losses, but were not sufficient for long-term graft survival in NHPs (Bottino et al. 2014).

5.4.3 Control of Complement and Coagulation, Prevention of Apoptosis

Depending on the transplantation site, activation of the complement and coagulation cascades may occur in pig-to-primate cell and tissue xenotransplantation and play a major role in IBMIR, humoral and cellular immune responses to the xenograft (see Sect. 3). Uncontrolled activation of these systems is a consequence of species incompatibilities between porcine regulatory proteins and the human and NHP complement and coagulation systems (Graham et al. 2011). Genetic engineering of donor pigs to express human complement-regulatory proteins, such as hCD46 (membrane cofactor protein; MCP), hCD55 (complement decay-accelerating factor,

DAF), hCD59 (membrane inhibitor of reactive lysis, MIRL) alone, or better in combination, can attenuate complement activation and prolong survival of pig-to-primate xenografts (reviewed in Cooper et al. 2016c).

Several strategies have been pursued to overcome coagulation dysfunction, including the generation of transgenic pigs expressing human thrombomodulin (THBD) (Wuensch et al. 2014), tissue factor pathway inhibitor (TFPI) (Lin et al. 2010), endothelial protein C receptor (EPCR) (Iwase et al. 2014), or ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1, CD39) (Wheeler et al. 2012) (reviewed in Klymiuk et al. 2016; Cowan et al. 2016). Moreover, transgenic pigs expressing antiapoptotic and anti-inflammatory proteins, such as human tumor necrosis factor-alpha-induced protein-3 (A20) (Oropeza et al. 2009) and human heme oxygenase-1 (HO-1) (Fischer et al. 2016; Petersen et al. 2011), have been produced.

A large proportion of pancreatic islets are lost due to apoptosis during isolation, culture and transplantation. Adenoviral transduction of human islets with X-linked inhibitor of apoptosis protein (XIAP), which blocks caspases 3, 7, and 9, dramatically reduced the number of human islets required for reversing hyperglycemia in diabetic immune-deficient mice (Emamaullee et al. 2005). Transgenic pigs that express XIAP under the control of the porcine *INS* promoter in pancreatic beta-cells have been generated, and islets are currently being tested in staurosporine-induced apoptosis assays and transplantation experiments with reduced islet numbers (reviewed in Klymiuk et al. 2016).

5.4.4 Local Immunomodulation to Improve Graft Acceptance

Controlling T-cell responses by inducing tolerogenic T cells while inhibiting cytotoxic T cells is key to long-term survival of xenografts such as islets. Blocking the CD80/CD86-CD28 co-stimulation pathway with CTLA4-Ig (abatacept®) or LEA29Y (belatacept®) has improved long-term allogeneic and xenogeneic graft outcome (reviewed in Bartlett et al. 2016). Xenotransplantation has the advantage that donor pigs can be engineered to express these factors, enabling inhibition of T-cell activation at the graft site without systemic immunosuppression of the recipient, reducing the risk of infection (commented in Aikin 2012). This has been successfully applied in immune-compromised humanized diabetic mice that received LEA29Y-expressing porcine neonatal islet-like cell clusters (Klymiuk et al. 2012a), and in further experiments using human CTLA4-Ig expressing corneal (Vabres et al. 2014) and skin xenografts (Wang et al. 2015). Transgenic pigs expressing LEA29Y under control of the CAG promoter in multiple tissues have been generated. Although these pigs are immunocompromised, they can be propagated by sexual reproduction and may serve as a source of other cell and tissue types that are protected against T-cell mediated rejection (Bahr et al. 2016).

As a complementary strategy, tolerogenic T-cell responses can be induced by transgenic overexpression of the co-inhibitory ligand PD-L1 (Plege et al. 2009; Plege-Fleck et al. 2014) (see also Sect. 3.3). Expression of human dominant-negative mutant class II transactivator (CIITA-DN) has been used to suppress SLA class II expression on porcine cells (Hara et al. 2013). Class I MHC deficient pigs have also

been generated and reported to have reduced levels of $CD4^-$ $CD8^+$ T cells in peripheral blood, but appeared healthy and developed normally (Reyes et al. 2014). Porcine cells and tissues expressing no or low levels of SLA should have particularly low immunogenicity for the human immune system.

Transgenic pigs expressing human TNF-related apoptosis inducing ligand (TRAIL) (Klose et al. 2005; Kemter et al. 2012), human FAS ligand (FASL) (Seol et al. 2010), and HLA-E/beta2-microglobulin have been generated to prevent cellular rejection of xenografts (Weiss et al. 2009). Cells from the latter pigs were effectively protected against human NK-cell-mediated cytotoxicity, depending on the level of CD94/NKG2A expression on the NK cells (Weiss et al. 2009). Human CD47 has been expressed on porcine cells to activate the "don't eat me" signal receptor SIRPα on (human) monocytes/macrophages and downregulate their phagocytic activity (reviewed in Cooper et al. 2016c; Meier et al. 2015).

5.5 Stimulation of Graft Vascularization

Efficient vascularization of xenoislets or other xenogeneic tissue is essential for their engraftment and long-term function. One way of achieving this is preconditioning of the transplantation site, for example, by creating a prevascularized, subcutaneous pouch into which islets are placed (Pepper et al. 2015). Genetically modified islets that express angiogenic factors are another possibility (reviewed in Klymiuk et al. 2016). After transplantation under the kidney capsule of syngeneic diabetic mice, transgenic mouse islets expressing vascular endothelial growth factor A (VEGFA) under the control of a rat insulin promoter showed enhanced microvascular density and functional blood flow into the grafts (Lai et al. 2005). Similar effects were observed after adenoviral transduction of mouse islets with a VEFGA expression cassette (Zhang et al. 2004). An inducible system based on the binary Tet-On system (Klymiuk et al. 2012b) has been adapted to enable beta-cell-specific inducible expression of VEGFA in pigs, and the re-vascularization capacity of islets from such donor pigs is being tested after xenotransplantation into the anterior chamber of the mouse eye (Speier et al. 2008a, b) and under the kidney capsule of diabetic mice (Klymiuk et al. 2012a).

5.6 Protection against Transmitting Pathogens by Xenogeneic Cells through Genetic Editing

The potential risks posed by porcine endogenous retroviruses (PERV) have long been seen as a major hurdle for clinical xenotransplantation of porcine tissues (Denner et al. 2009), but recent data from studies in NHPs and humans (Wynyard et al. 2014) have not indicated any PERV transmission. Nonetheless, the presence of endogenous viruses in xenografts is a source of unease and various strategies have been devised to address this, including donor animals with naturally low

PERV expression and knocking down PERV expression by transgenic techniques (reviewed in Denner and Mueller 2015). The number of PERV loci present in the porcine genome makes their complete removal unrealistic, but recently all PERV integrants in a porcine cell line have been disrupted by gene editing with CRISPR/Cas9. This was achieved using guide RNAs targeting the retroviral pol gene, which is highly homologous in PERV A, B, and C (Yang et al. 2015b), opening the prospect of PERV-free donor pigs that in fact were generated recently (Niu et al. 2017).

5.7 Functional Improvement of Xenogeneic Cells by Genetic Engineering of Donor Pigs

The great majority of genetic modifications to xenodonor pigs have been designed to overcome immune rejection. However, porcine islets also have the problem of lower insulin secretory activity and response to stimuli than healthy islets from NHPs or humans.

To overcome this, Gianello and co-workers (Mourad et al. 2015; reviewed in Cooper et al. 2016a) have investigated means of increasing the activity of pathways that amplify the increase in cytosolic calcium in beta-cells triggered by glucose and required for insulin granule exocytosis. These pathways are:

(i) A cAMP-dependent pathway, activated by binding of glucagon-like peptide-1 (GLP1) to its G-protein-coupled receptor on beta-cells and leading to activation of protein kinase a
(ii) A cholinergic pathway, activated by binding of acetylcholine or cholecystokinin to a type 3 muscarinic receptor (M3R)

Both pathways increase the number of readily-releasable insulin granules in beta-cells and result in a greater secretory response to glucose stimulation (reviewed in Cooper et al. 2016a). Mourad et al. (2015) demonstrated that GLP1 and M3R expressed from viral vectors in beta-cells act synergistically to enhance insulin secretion by adult porcine islets and NICCs. Plans are underway to extend this concept to transgenic pigs (reviewed in Klymiuk et al. 2016).

6 Types of Porcine Cells and Tissues for Xenogeneic Transplantation

Various porcine cell types and tissues have been tested in preclinical xenotransplantation experiments and clinical trials. The following sections summarize the state of the art and the challenges that must be overcome to realize clinical application.

6.1 Porcine Islets to Cure Type 1 Diabetic Patients with a Labile Metabolic Condition

In 2014, the first "Global Report on Diabetes" of the World Health Organization (WHO) (http://www.who.int/diabetes/global-report/en/) estimated that more than 422 million people worldwide suffer from diabetes. In children this is predominantly type 1 diabetes (T1D). T1D is caused by irreversible immune-mediated destruction of insulin-producing pancreatic beta-cells and requires lifelong insulin replacement therapy. In 2015, 542,000 children worldwide were estimated to suffer from type 1 diabetes (T1D) (http://www.diabetesatlas.org/). The incidence and prevalence of T1D vary between continents, high in USA and Europe and low in India and China, and even between neighboring areas. For example, Finland has more than 60 cases per 100,000 each year, but its neighbor Estonia has less than one third of these cases (Atkinson et al. 2014) (http://www.who.int/diabetes/global-report/en/). Overall, the global incidence of T1D has been increasing for decades.

Diabetes care has made remarkable advances in recent years, with technical developments such as continuous glucose monitoring and (closed-loop) insulin pumps (Stewart et al. 2016), and new and improved drugs such as insulin analogues, incretins, and other hormones (reviewed in Atkinson et al. 2014). However, these therapeutic options are insufficient for T1D patients with frequent hypoglycemic episodes and extreme glycemic lability, so-called "brittle disease." For these patients, pancreas or islet transplantation is the best option (reviewed in Choudhary et al. 2015), but the shortage of appropriate human donor pancreata and islets severely limits the availability of such treatment (Bruni et al. 2014). Porcine islets could meet this demand (Reichart et al. 2015) and several advantages, including the functionality of porcine insulin in humans due to high similarity in protein sequence, support the pig as a donor species (reviewed in Klymiuk et al. 2016).

Porcine islets or islet cells have been isolated from different developmental stages ranging from embryonic pancreatic primordia to pancreata from retired breeding animals at age several years (reviewed in Nagaraju et al. 2015; Korbutt 2008).

Islets from retired breeders or young adult animals are functionally mature and structurally fully developed, but may be rather fragile. Especially retired female breeders of large size yield high numbers of large compact islets that function shortly after transplantation (reviewed in Nagaraju et al. 2015). However, the isolation of adult islets is technically demanding and expensive, and long-term maintenance of the donor animals under designated pathogen-free (DPF) conditions requires considerable investment in infrastructure and incurs high running costs. Based on work in NHP models, it has been estimated that several pigs may be required to provide enough islets to achieve normoglycemia in a 70 kg human. The actual number may however be smaller since glucose metabolism in humans is more similar to pig (reviewed in Nagaraju et al. 2015).

Islet-like cell clusters (ICCs) from neonatal or fetal porcine pancreata are easier to isolate than adult islets. After enzymatic digestion of pancreas tissue and culture for several days, only the endocrine ICCs survive and can be harvested. However, ICCs do not function immediately, i.e., they require maturation in vitro and after

transplantation in vivo. In diabetic NHPs, normoglycemia was achieved 3–4 weeks after intraportal transplantation of neonatal ICCs (NICCs) (Cardona et al. 2006; Thompson et al. 2012). For ICCs isolated from mid-term fetal pancreas, the delay to restore normoglycemia in diabetic mice was 2–6 months (Korsgren et al. 1991). A critical point is the large number of neonatal pigs or fetuses required as pancreas donors per patient. Estimates range between 70 and 100 for a 70 kg patient (Korbutt et al. 1996). The advantage of using NICCs is that the donor animals can be transferred into a DPF facility via cesarean section and only need to be maintained for a short period, a few days to 1 month at most. Moreover, an efficient and scalable protocol has been developed for the isolation of NICCs from multiple neonatal pancreata (Ellis et al. 2016).

Another option is the use of porcine embryonic pancreatic tissue. The main advantage is that after transplantation only endocrine cells with a predominance of beta-cells are formed (reviewed in Hammerman 2013). Embryonic precursor tissue also exhibits remarkable growth potential, eventually adapting in size to the recipient organism (discussed in Hecht et al. 2009). After transplantation of primordial pancreatic tissue from porcine embryos at day 28 of development (E28) into the mesentery of nonimmunosuppressed STZ-diabetic rhesus macaques, engraftment of porcine beta-cells in liver, pancreas, and mesenteric lymph nodes was demonstrated, and insulin requirements were reduced in one macaque followed over 22 months posttransplantation (Rogers et al. 2007). A subsequent study (Hecht et al. 2009) used E42 porcine pancreatic tissue, which had previously been shown to be superior to E28 in terms of growth potential and insulin secretion (Eventov-Friedman et al. 2006), for implantation into the omentum of STZ-diabetic cynomolgus monkeys. Two recipients, followed for 280 and 393 days, demonstrated that intra-omental implantation of E42 porcine embryonic pancreatic tissue restores an enduring euglycemic state. However, immunosuppression was required and it took 2–3 months before the graft became fully functional. Extrapolation from this study indicated that pancreas tissue from around 60 E42 porcine fetuses would be needed to restore blood glucose control in an adult diabetic patient (Nagaraju et al. 2015). The logistic problems of recovering such a large number of exactly staged porcine embryos and the delay in onset of function make the use of embryonic tissue less attractive for clinical development than NICCs and adult islets (Nagaraju et al. 2015).

Regarding methods of delivering islets into the recipient, intraportal transplantation, as performed in allotransplantation, is currently preferred for free porcine islets (wild-type or genetically modified), intraperitoneal sites (omental pouches) for microencapsulated islets, and subcutaneous sites for macroencapsulation devices. Intramuscular implantation of free islets is currently under investigation (Dufrane et al. 2010; Ludwig et al. 2015; Shin et al. 2015; Wolf-van Buerck et al. 2015).

There are still major immunological obstacles to successful clinical islet xenotransplantation (see Sect. 3), but strategies to overcome these have been developed (see Sect. 5). No immunosuppression of recipients receiving micro- or macroencapsulated wild-type porcine islets is required. Xenotransplantation of free islets does however require immunosuppression or the use of genetically modified donors

to avoid IBMIR, humoral, and cellular rejection. Some genetic modifications, e.g., elimination of proinflammatory molecules or transgenes to improve survival and function, may also be beneficial for encapsulated islets (reviewed in Klymiuk et al. 2016).

Remarkable progress has been made in restoring glucose control by transplanting wild-type porcine islets into diabetic NHPs with immunosuppression (reviewed in Klymiuk et al. 2016; Bartlett et al. 2016). In 2006, two publications reported long-term maintenance of normoglycemia after intraportal transplantation of porcine NICCs (about 6.2×10^6 beta-cells/kg body weight) into pancreatectomized rhesus macaques (Cardona et al. 2006), or adult pig islets (25,000 islet equivalents/kg; cultured for 48 h) into STZ-induced diabetic cynomolgus macaques (Hering et al. 2006). Massive immunosuppression of the recipients was necessary, including CD154-specific monoclonal antibody treatment, which would not be clinically possible due to thrombogenic side effects.

Thompson et al. (2012) treated STZ-induced diabetic rhesus macaques with intraportal infusions of ~50,000 islet equivalents/kg wild-type neonatal porcine islets. Maintenance immunosuppression of cohort 1 included LEA29Y (belatacept®) and mycophenolate mofetil (MMF) plus induction with basiliximab® (IL2 receptor inhibitor) and lymphocyte function-associated antigen-1 (LFA-1) blockade. Cohort 2 had additional tacrolimus induction. Cohort 3 received alefacept® (LFA-3 blockade) instead of basiliximab® and a more intense LFA-1 blockade. In contrast to cohort 1, cohorts 2 and 3 achieved sustained insulin-independent normoglycemia (median rejection-free survival times 60 and 111 days) demonstrating that sparing the CD40/CD154 pathway blockade is possible.

In a more recent study, Shin et al. (2015) introduced adult wild-type porcine islets intraportally into STZ-induced diabetic rhesus macaques. Immunosuppressive induction included cobra venom factor (CVF), anti-thymocyte globulin (ATG), anti-TNFα antibody (adalimumab®) and was maintained with anti-CD154 monoclonal antibody and low-dose sirolimus. The islet grafts survived and were able to control glucose homeostasis for 167, 180, 303, 512, and more than 603 days.

In spite of these promising results, translation into clinical application is hindered by the large number of islets required and the need for immunosuppression. As mentioned previously, anti-CD154 monoclonal antibody is not clinically useful because it is thrombogenic in humans (Kawai et al. 2000).

The need for systemic immunosuppression can however be reduced or even avoided by encapsulation (see Sect. 5.2), or genetic modification of donor pigs (see Sect. 5.4).

Inactivation of *GGTA1* leading to αGal deficiency did not affect the long-term outcome of intraportally transplanted adult pig islets (van der Windt et al. 2009), presumably due to the naturally low level of αGal on adult islet cells (Rayat et al. 2003). Expression of hCD46 had no effect on adult islet loss in the early post-transplant period, but was beneficial for long-term survival (van der Windt et al. 2009). In STZ-induced diabetic rhesus monkeys with immunosuppression, knockout of *GGTA1* was advantageous for survival and engraftment of NICCs, which would otherwise express high levels of αGal (Thompson et al. 2011). Additional expression

of hCD55 and hCD59 further attenuated IBMIR after intraportal transplantation into nondiabetic baboons (Hawthorne et al. 2014). Data from an elegant "dual islet transplant model," which allows comparison of two different islet preparations within the same recipient, showed that in the absence of immunosuppression, a robust inflammatory response may precede IBMIR, masking the beneficial effect of *GGTA1* knockout observed in previous studies (Martin et al. 2015).

T-cell mediated rejection is a major barrier for long-term survival of islet xenografts. Klymiuk et al. (2012a) have generated transgenic pigs expressing the T-cell co-stimulation blocking molecule LEA29Y under control of the porcine insulin promoter. After implantation under the kidney capsule of diabetic NOD-SCID $Il2rg^{-/-}$ (NSG) mice, LEA29Y expressing NICCs restored glucose control and, in contrast to wild-type NICCs, were not rejected by transplanted human blood mononuclear cells (PBMCs) (Fig. 3). Importantly, only very low levels of LEA29Y were detectable in the circulation of mice grafted with transgenic islets, supporting the concept of local immune modulation by LEA29Y (Klymiuk et al. 2012a). These findings have recently been extended in diabetic NSG mice grafted with human $CD34^{+}$ hematopoietic stem cells, where LEA29Y transgenic islets survived and maintained glucose control for more than 6 months (Wolf-van Buerck et al. 2017).

A few clinical studies have been performed using porcine islets, some of which are ongoing (Matsumoto et al. 2016; Cooper et al. 2016a; Fotino et al. 2015; Wynyard et al. 2014; Nagaraju et al. 2015). In 2007, Living Cell Technologies (LCT) launched a phase I/IIa trial in Russia using wild-type NICCs microencapsulated within alginate/poly-L-ornithine/alginate (DIABECELL®) (reviewed in Dufrane and Gianello 2012; Yang and Yoon 2015). Seven patients with insulin-dependent diabetes received between one and three implants of DIABECELL® (5000 and 10,000 IEQ/kg). Blood glucose levels improved in five of seven patients, and two patients became insulin independent for up to 32 weeks. Following the successful completion of this trial, three phase I/II studies have been launched in New Zealand and Argentina, incorporating an improved transplantation protocol (reviewed in Cooper et al. 2016a; Yang and Yoon 2015). In the Argentina study, nonimmunosuppressed patients with unstable T1D received two doses of 5000 IEQ/kg ($n = 4$), or 10,000 IEQ/kg ($n = 4$) microencapsulated wild-type preweaned porcine islet-like cell clusters intraperitoneally, with the second transplant carried out 3 months after the first. Patients receiving 10,000 IEQ/kg twice showed reduction of glycated hemoglobin (HbA1c) $<7\%$ for more than 2 years (reviewed in Cooper et al. 2016a). Patients also exhibited significantly fewer unaware hypoglycemic episodes.

6.2 Corneal Xenografts to Cure Corneal Blindness

Corneal diseases, which occur in most developing countries, are estimated to be the second leading cause of blindness worldwide (Oliva et al. 2012). Cure of blindness and visual impairment is of huge socio-economic importance (reviewed in Lamm et al. 2014). Corneal transplantation (keratoplasty) restores visual function and is estimated to be the most common transplant performed (Gain et al. 2016). There is

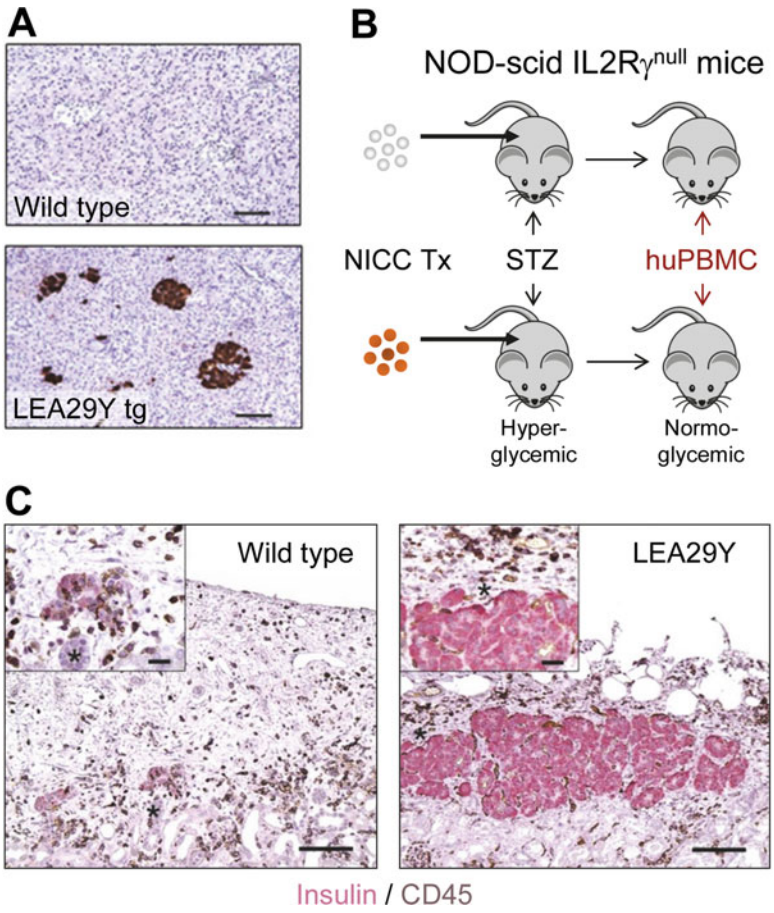


Fig. 3 Expression of LEA29Y in porcine islets prevents rejection by human T cells. (**a**) Immunohistochemical staining of LEA29Y in the islets of INS-LEA29Y transgenic (tg) pigs. (**b**) Neonatal islet-like cell clusters (NICCs) from wild-type and LEA29Y tg piglets were transplanted (Tx) under the kidney capsule of immunodeficient, STZ-diabetic mice. Islets of both genotypes restored normoglycemia after several weeks. Then the recipients were reconstituted with human peripheral blood mononuclear cells (huPBMC). (**c**) Histological sections of the transplantation site of wild-type or LEA29Y transgenic islets at the end of the observation period (4 weeks after Tx). Insulin positive cells are stained in pink, T cells in brown. Note that after transplantation of wild-type NICCs, only few insulin-positive cells are left, but a strong T-cell infiltration can be observed. After transplantation of LEA29Y tg NICCs large clusters of insulin positive cells remain and T-cell infiltration is only observed in their periphery. Bars: 100 μm, in insets 20 μm. (From Klymiuk et al. 2012a)

however a severe shortage of grafts, with only one cornea available for 70 needed, and alternatives to allografts are urgently required. Porcine cornea is a potentially attractive option due to comparable refractive power and plasticity, similar size, and tensile strength as the human tissue (Hara and Cooper 2011; Kim et al. 2011).

Humoral and cellular immune responses limit corneal xenograft survival and function, but can be addressed by genetic modification of the donor pigs. The International Xenotransplantation Association (IXA) recently published a consensus statement on conditions for undertaking clinical trials of xenocorneal transplantation regarding specific ethical, logistical, scientific, and regulatory issues and proposed guidelines (Kim et al. 2014; see also Sect. 7).

Depending on the extent of the corneal disease, several surgical techniques may be applied, including penetrating keratoplasty (PK), anterior lamellar keratoplasty (ALK), and endothelial keratoplasty (EK) (reviewed in Lamm et al. 2014). Porcine corneal xenografts for ALK, which involves transplantation of the anterior epithelium and stroma layers of the cornea but not Descemet's membrane or endothelium, have survived longer (>3 months) in rhesus monkeys than xenografts containing corneal endothelium, as used for PK and EK. This is because the immune response seems mainly directed against corneal endothelial cells.

The cornea is normally an immune privileged tissue, since blood and lymphatic vessels are absent, MHC molecules are weakly expressed, PD-L1 and FASL inhibiting T-cells are abundant, and numerous immune modulating molecules are present in the aqueous humor (reviewed in Hara and Cooper 2010). However, pathological conditions may lead to loss of immune privilege due to inflammation and neovascularization. The main cause of corneal allo- and xenograft rejection weeks to months after transplantation is a T-cell response; macrophages may also contribute. Humoral immune responses are particularly relevant in high-risk patients with an inflamed or neovascularized corneal bed, which is the case in 30% of recipients.

Strategies to prevent cornea xenograft rejection include reduction or elimination of xenoantigens, especially Neu5Gc and αGal, and suppression of the T-cell response. Deletion of Neu5Gc and αGal antigens plus expression of human complement regulatory proteins (e.g., hCD46) has been suggested as an effective approach. In vitro results have demonstrated a reduced humoral and cellular immune response to corneal cells of αGal deficient, hCD46 transgenic, and αGal deficient, hCD46/CIITA-DN double-transgenic pigs compared to wild-type corneas (reviewed in Kim and Hara 2015). However, in one pig-to-NHP study, corneas of αGal deficient, hCD39/hCD55/hCD59/fucosyl-transferase transgenic pigs were not superior to wild-type pigs (Vabres et al. 2014). Notably these corneas still had the major xenoantigen Neu5Gc.

Long-term survival (up to >900 days) of full-thickness PK corneal grafts of wild-type donor pigs in NHPs has been achieved by blockade of the CD40-CD154 T-cell costimulatory pathway with anti-CD154 monoclonal antibody (mean survival >300 days in anti-CD154 mAb treated group vs. 28 days for controls receiving immunosuppressive regime without anti-CD154 mAb) (Choi et al. 2015). Expression of a human CTLA4-Ig transgene in keratocytes extended cornea xenograft survival threefold after ALK compared to wild-type (Vabres et al. 2014).

A recent clinical trial investigated treatment of fungal corneal ulcers with acellular porcine corneal stroma (APCS) (Zhang et al. 2015). All ulcers healed with the return of neovascularization, epithelialization occurred in 43 of 47 grafts, and

41 grafts gradually became transparent without rejection, resulting in significant visual improvement. The authors concluded that APCS grafts are safe and effective for treating corneal fungal ulcers and potentially other corneal lesions.

6.3 Xenogeneic Neuronal Cells for the Treatment of Neurodegenerative Diseases

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by loss of dopaminergic neurons in the substantia nigra of the brain (reviewed in Hague et al. 2005). Huntington's disease (HD) is an autosomal dominant hereditary disease caused by mutations in the Huntingtin (*HTT*) gene that cause abnormal expansion of a glutamine stretch in the N-terminal protein sequence (reviewed in Saudou and Humbert 2016). HD is characterized by gradual neuron death and alterations in dopamine and dopamine receptor levels in the brain (Gardoni and Bellone 2015).

Transplantation of neuronal cells into adults is possible. Transplanted fetal neurons were shown to survive in the recipient's brain (Lindvall et al. 1990; Freeman et al. 2000). However, ethical and regulatory issues, risk of infection, and limited availability restrict the use of human fetal neurons for transplantation (Vadori et al. 2015). Fink et al. (2000) have summarized preliminary results of phase I studies of porcine fetal neuronal cell xenotransplantation into the brains of PD and HD patients and reported some clinical improvement. Recently, porcine neuroblasts transplanted in an NHP model of Parkinson's disease were shown to differentiate into mature dopaminergic neurons and to improve clinical symptoms (Aron Badin et al. 2016). Although the brain is considered an immune-privileged transplantation site and fetal-derived tissue is less immunogenic than tissue from adult donors, T-cell mediated transplant rejection and a humoral immune response can occur (Vadori et al. 2015). In a recent pig-to-primate xenotransplantation study, the combination of systemic and local intracerebral immunosuppression improved neuronal graft survival (Aron Badin et al. 2016). Local inhibition of T-cell co-stimulation by neuronal cells from transgenic fetuses expressing human CTLA4-Ig under the control of a neuron-specific enolase promoter was tested in an NHP model of Parkinson's disease. In combination with systemic immunosuppression (cyclosporine, mycophenolate and prednisone), these showed longer survival and better restoration of locomotor functions than wild-type neurons (Aron Badin et al. 2016).

6.4 Porcine Skin Grafts for Patients with Major Skin Burns or Chronic Wounds

Autologous skin transplantation is the only option to achieve permanent closure of massive wounds with complete restoration of skin function (Zhou et al. 2013). If sufficient autologous skin graft is not available, e.g., in patients with major burns or chronic refractory wounds, allogeneic skin is used as a temporary biological wound

dressing. However, the need far exceeds the tissue available. Cadaveric skin allografts are widely used in plastic surgery, but costs are high, availability is limited, and they could transmit pathogens (Rowan et al. 2015). Although numerous alternative synthetic and biological wound dressings have been developed, skin transplantation is superior. Allogeneic and xenogeneic skin grafts both promote re-epithelialization of wounds, and the healing rate is better and the costs of wound care are lower than with traditional dressings (Hermans 2011).

Co-transplantation of autologous split-thickness skin with porcine acellular dermal matrix into full-thickness wounds caused by burn or trauma was recently shown to be superior to autologous split-thickness skin grafting alone (Jiong et al. 2010; Chen et al. 2013). Grafts with xenogeneic dermal matrix formed a structure similar to normal skin, including sweat gland-like structures, and inhibited scar formation. Xenogeneic skin grafts with viable cells have the advantage of availability and biological activity (Zhou et al. 2013). Porcine skin is favored because it resembles human skin in dermal tissue structure, collagen arrangement, and follicle density (MacLeod et al. 2004). However, before entering the clinic, genetic modification will be necessary to overcome humoral and cell-mediated immune rejection (see Sect. 5.4).

Skin xenografts from αGal-deficient pigs showed prolonged survival in baboons, comparable with that of skin allografts (Weiner et al. 2010). Albritton et al. (2014) also describe grafting αGal-deficient porcine skin grafts onto baboons. The grafts were finally rejected by an antixenogeneic humoral response, but, importantly, recipients showed no sensitization to alloantigens, nor accelerated rejection of subsequent allogeneic skin grafts. The authors concluded that αGal-deficient porcine skin grafts can be used as a first-line treatment of severe burns and do not preclude subsequent skin allotransplantation (Albritton et al. 2014).

To overcome T-cell mediated rejection of porcine skin xenografts, donor pigs have been genetically engineered to express CTLA4-Ig under control of the human keratin 14 gene promoter. Skin from these transgenic pigs xenografted onto rats exhibited remarkably prolonged survival compared to wild-type grafts (Wang et al. 2015). Tena et al. (2016) have attempted to induce tolerance in a pig-to-baboon skin transplantation model by mixed hematopoietic chimerism. To overcome the problem that porcine hematopoietic stem cells are rapidly cleared by primate macrophages, they generated CD47 transgenic pigs and used mobilized peripheral blood hematopoietic cells expressing high levels of human CD47, which inhibits phagocytosis via binding to SIRPα receptors on macrophages. Split thickness skin grafts from the hematopoietic cell donor swine were grafted onto baboons 5 weeks after the last cell infusion and 7 weeks after discontinuing all immunosuppression, to test the immune response. The level and duration of transient chimerism were substantially increased in baboons that received human CD47-expressing porcine hematopoietic cells. Skin graft survival on high CD47 recipients was also prolonged, in one case there were no signs of rejection at least 53 days after placement. The authors concluded that prolongation of transient porcine chimerism by expression of human CD47 has an immune modulating effect that markedly prolongs survival of pig-to-baboon skin xenografts.

6.5 Xenogeneic Hepatocytes to Overcome Hepatic Failure or Enzyme Defects

The liver plays a central role in carbohydrate, protein, amino acid, and lipid metabolism. Furthermore, this organ synthesizes coagulation factors and acute phase proteins and is crucial for drug metabolism and detoxification. In severe acute liver failure and end-stage liver failure, liver transplantation may be the only life-supporting therapy (reviewed in Zarrinpar and Busuttil 2013). In view of the severe shortage of human donor organs, hepatocyte transplantation might be a promising alternative to whole-organ liver transplantation, or at least serve as a bridge to liver transplantation (Meier et al. 2015; Dhawan et al. 2010). Allotransplantation of hepatocytes has given encouraging results in the clinic and has also been used in patients with metabolic defects of the liver where the hepatocyte graft provides the missing enzyme or protein (Hughes et al. 2012).

Xenotransplantation of hepatocytes has numerous potential advantages, including only moderate invasiveness of the transplant procedure, low morbidity and high safety, a potentially unlimited supply of cells, the option to protect the cells by encapsulation, the use of genetically modified cells, and the possibility of cryopreserving hepatocytes (Meier et al. 2015 and references therein). The first clinical application of porcine hepatocytes is likely to be in patients with acute fulminant liver failure requiring organ replacement. The hepatocyte xenograft would provide temporary liver support to stabilize metabolic functions while the patient waits for an allotransplant or the regeneration of their own liver.

Porcine and human hepatocytes have numerous metabolic similarities (Kobayashi et al. 1998; Soucek et al. 2001; Ekser et al. 2012b). Nevertheless, there are important species differences in some components of the coagulation system, for example, in the regulation of fibrin synthesis, and the expression pattern or abundance of important proteins such as cytochrome P450, albumin and C-reactive protein (Ramackers et al. 2014; Kleine et al. 2008; Schrem et al. 2006). These differences will have to be modified by genetic modification of donor cells, or corrected by therapeutic intervention while xenografted hepatocytes provide temporary liver support.

In the first successful xenotransplantation of this kind, porcine hepatocytes in 1% alginate solution were infused into the spleen of cynomolgus monkeys with intact liver function. Xenograft function, as measured by porcine albumin in the recipients' circulation, was detectable for more than 80 days after a single infusion and more than 8 months after repeated infusion. Recipient monkeys were treated with a systemic immunosuppression regimen similar to that used in human organ allotransplantation (Nagata et al. 2007).

Hepatocytes can be protected against humoral attack by the recipient's complement and innate humoral immune system by microencapsulation in alginate-poly (L-lysine)-alginate microspheres (Meier et al. 2015 and references therein). Transplantation of microencapsulated porcine hepatocytes in baboons with induced fulminant liver failure substantially improved survival and biochemical parameters (reviewed in Meier et al. 2015). IBMIR can occur if native hepatocytes contact

blood flow, as in the case of portal vein blood flow, leading to their death. This applies to allogeneic or xenogeneic hepatocytes (Gustafson et al. 2011), both of which express high levels of procoagulant tissue factor (Stephenne et al. 2007). Genetic modifications of donor hepatocytes might be necessary for prolonged graft acceptance (see Sect. 5.4 and Meier et al. 2015).

6.6 Xenogeneic Chondrocytes for the Treatment of Degenerative Disk Disease and Articular Cartilage Lesions

Articular cartilage defects are common in orthopedic practice. There are hopes of improving long-term outcomes by tissue engineering techniques using transplanted living chondrocytes to repopulate and repair cartilage defects by cartilage synthesis (Mollon et al. 2013 and references therein). Cell-based treatments, such as autologous chondrocyte implantation and mesenchymal stem cell implantation, are already used in the clinic as a better option for cartilage treatment than cell-free methods (Deng et al. 2016). However, a large number of chondrocytes are required to achieve hyaline cartilage synthesis and healing of cartilage lesions (Mollon et al. 2013 and references therein). The use of porcine chondrocytes, which are abundantly available, is currently hindered by humoral and cellular immune responses to xenogeneic cartilage and complement activation, causing graft loss within weeks (Stone et al. 1997; Sommaggio et al. 2013). Further progress will require genetic modification of donor pigs to address critical factors involved in graft loss, including immunogenic epitopes such as αGal, surface expression of the immunoregulatory proteins CD86 and VCAM-1 on pig chondrocytes, and the deleterious effects of complement activation (Sommaggio et al. 2009, 2013; Costa et al. 2003).

6.7 Red Blood Cells for Clinical Transfusion

Red blood cell (RBC) transfusion is carried out to rapidly increase the supply of oxygen to tissues and is indicated in patients with acute anemia, chronic anemia that has failed to respond to other treatments and to treat inherited blood disorders, such as thalassemia or sickle cell anemia (Carson et al. 2012). Approximately 85 million units of RBCs are transfused annually worldwide. Porcine erythrocytes could potentially solve the shortage of human RBCs for transfusion. As with human RBC transfusion, xeno-RBCs must be free of antigens that bind to human preformed antibodies to avoid antibody-mediated complement lysis and to minimize removal by macrophages (Cooper et al. 2010). Genetic deletion of the αGal and Neu5Gc xenoantigens in the donor pigs significantly reduces human antibody binding and antibody-mediated complement lysis in vitro (Wang et al. 2014).

6.8 Testicular Sertoli Cells as Immune Modulators and Cell-Based Therapies

Testicular Sertoli cells (SCs) are key players in conferring immune privilege to the testis. Foreign tissue grafts can survive in the testis for extended periods because immune surveillance is attenuated and immune response is greatly reduced (reviewed in Mital et al. 2010). Co-transplantation of SCs with allo- and xenogeneic cells, including pancreatic islets and neurons, at sites outside the testis can prolong graft survival and induce graft acceptance in the absence of systemic immunosuppression by creating an ectopic immune-privileged environment. This seems to be mediated by SC-derived immunomodulatory factors, such as complement inhibitors, cytokines, and inhibitors of cytotoxic lymphocytes (reviewed in Mital et al. 2010; Luca et al. 2015).

Xenotransplantation into naïve Lewis rats demonstrated that neonatal porcine SCs inhibit both the alternative and classical pathways of complement-mediated cell lysis, while porcine NICCs were rejected mainly via the alternative pathway (Wright et al. 2016). A study of porcine SCs co-transplanted subcutaneously with porcine NICCs in a stainless steel wire mesh chamber in 12 insulin-dependent diabetic patients in the absence of immunosuppressive treatment found that six of the 12 patients had significant reductions in exogenous insulin requirements for at least 4 years posttransplant, while maintaining stable or improved HbA1c levels (Valdes-Gonzalez et al. 2005, 2007; reviewed in Mital et al. 2010). Several critical parameters affecting Sertoli cell-mediated immune protection were identified (reviewed in Mital et al. 2010):

(i) Number of SCs co-transplanted with the NICCs
(ii) Age of the SC donor
(iii) Purity of the isolated SC preparation
(iv) Culture of SCs before transplantation

Regarding the latter point, it was shown that culture of isolated SCs for 48 h leads to formation of cell aggregates with differentiated and polarized SCs, resulting in improved SC-mediated immune protection and survival of co-transplanted cells (reviewed in Mital et al. 2010).

Interestingly, it may not be necessary to co-transplant SCs with the graft, because microencapsulated porcine SCs placed in the peritoneal cavity significantly prolonged survival of allogeneic skin grafts in a rodent model (Bistoni et al. 2012). This effect was mediated by Sertoli cell-induced alterations to the host's immune system, as demonstrated by a significantly increased proportion of regulatory T cells (Tregs).

In addition to inducing graft tolerance, microencapsulated porcine SCs have been used to provide other therapeutic effects. Laron syndrome is a rare human disorder caused by defects in the growth hormone receptor (*GHR*) gene, resulting in irreversibly retarded growth. Recombinant human insulin-like growth factor-1 (IGF1) is currently the only available treatment option (Luca et al. 2013). A single intraperitoneal graft of microencapsulated porcine SCs producing porcine IGF1 successfully promoted

significant proportional growth in a mouse model of Laron syndrome. Experimentally induced type 1 diabetes in nonobese diabetic (NOD) mice has been prevented and reverted by transplantation of microencapsulated porcine SCs. The authors report that systemic immune tolerance was restored by TGFβ-dependent emergence of autoantigen-specific regulatory T cells and beta-cell function was recovered in the diabetic recipients (Fallarino et al. 2009). Intraperitoneal injection of microencapsulated porcine SCs into two rhesus macaques with type 2 diabetes (T2DM) and insulin resistance, resulted in slight improvement of the T2DM phenotype, which the authors ascribe to modulation of the adaptive immune system by the SCs (Luca et al. 2014).

6.9 Mesenchymal Stromal Cells to Facilitate Graft Re-Vascularization and Immune Modulation

Mesenchymal stromal cells (MSCs, also called mesenchymal stem cells) have regenerative, anti-inflammatory, and immune modulatory properties (reviewed in Li et al. 2014). Improved vascularization of a macroencapsulated islet device in NHPs was demonstrated when porcine islets were co-transplanted with MSCs (Veriter et al. 2014; see Sect. 5.2.2). Porcine MSCs (pMSCs) function across species barriers and might have considerable therapeutic potential, particularly in xenotransplantation. Proposed advantages of pMSCs in this context include:

(i) pMSCs can be obtained from the same individual pig used as the organ or cell donor, or from an identical (cloned) pig
(ii) They can be easily recovered in large numbers without prolonged ex vivo expansion
(iii) They can be obtained from genetically engineered pigs lacking major xenoantigens and/or expressing specific proteins supporting xenograft survival and function

Reduced immunogenicity of genetically modified pMSCs has been demonstrated in vitro. Possible mechanisms include induction of apoptosis or anergy in human T cells, or switching of the T-cell phenotype with induction of regulatory T cells.

7 Regulatory Aspects of Cell and Tissue Xenotransplantation

The regulatory aspects of clinical xenotransplantation are complex and comprehensive coverage is outside the scope of this chapter. The interested reader is referred to recent review articles on this topic (Gonzalez 2012; Schuurman 2015). In the following, we summarize a few key points:

- Xenogeneic cell therapies are regulated as Cell-Based Medicinal Products (CBMPs).
- Acellular products, such as heart valves and decellularized cornea, are not considered to be CBMPs, but rather medical devices.

- In Europe, CBMPs are regulated as Advanced Therapy Medicinal Products (ATMPs) by Regulation (EC) No 1394/2007 of the European Parliament and of the Council and amending Directive 2001/83/EC and Regulation (EC) No 726/2004 (EC 2007).
- In the United States, CBMPs are regulated as Cellular and Gene Therapy Products (FDA).
- If cells from genetically modified donor animals are involved, specific regulatory documents, such as Directive 2001/18/EC of the European Parliament and of the Council on the Deliberate Release into the Environment of Genetically Modified Organisms and repealing Council Directive 90/220/EEC (EC 2001), Guideline EMA/CAT/GTWP/671639/2008 on Quality, Non-Clinical and Clinical Aspects of Medicinal Products Containing Genetically Modified Cells (EMA 2012), or the FDA Guidance for Industry, Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs (FDA 2015), need to be taken into account.

Managing the risk of transmitting known and unknown pathogens is a major regulatory issue. Several regulatory authorities have established safety guidelines and requirements that xenogeneic cell-based medicinal products must fulfill. Examples are:

- Guideline EMEA/CHMP/CPWP/83508/2009 on Xenogeneic Cell-Based Medicinal Products (EMA 2009)
- Guidance for Industry, Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans' of the US Food and Drug Administration (FDA 2016)

IXA has recently published updated consensus statements on national regulatory frameworks pertinent to clinical islet xenotransplantation (Cozzi et al. 2016), strategies to prevent zoonotic infection from xenografts (Spizzo et al. 2016), genetic optimization of donor pigs (Cowan et al. 2016), porcine islet product manufacturing and release testing criteria (Rayat et al. 2016), preclinical efficacy and complication data required to justify a clinical trial (Cooper et al. 2016d), recipient monitoring and a response plan for preventing disease transmission (Denner et al. 2016), and patient selection for pilot clinical trials of islet xenotransplantation (Hering and O'Connell 2016). In addition there is an IXA consensus statement on conditions for undertaking clinical trials of xenocorneal transplantation (Kim et al. 2014).

8 Conclusion

Major progress in recent years clearly indicates that xenogeneic cell and tissue transplantation may become a clinically relevant option for the treatment of degenerative diseases or severe traumatic lesions. Novel genetic engineering techniques facilitate the generation of donor pigs to provide cells and tissues tailored for specific applications. Since all cells of a genetically pig have the same genetic makeup, this

approach is inherently safer than the use of virally transduced auto- or allogeneic cells. In addition, maintenance of the donor pigs in designated pathogen-free facilities minimizes the risk for transmission of infectious diseases.

Acknowledgments Our work on xenotransplantation is supported by the Deutsche Forschungsgemeinschaft (TRR127 "Biology of Xenogeneic Cell, Tissue and Organ Transplantation – from Bench to Bedside") and by the German Center for Diabetes Research (DZD). The authors are members of EU COST Action BM1308 "Sharing Advances on Large Animal Models – SALAAM."

References

Aikin RA (2012) How to kill two birds with one transgenic pig. Diabetes 61(6):1348–1349. https://doi.org/10.2337/db12-0201

Albritton A, Leonard DA, Leto Barone A, Keegan J, Mallard C, Sachs DH, Kurtz JM, Cetrulo CL Jr (2014) Lack of cross-sensitization between alpha-1,3-galactosyltransferase knockout porcine and allogeneic skin grafts permits serial grafting. Transplantation 97(12):1209–1215. https://doi.org/10.1097/TP.0000000000000093

Al-Mohanna F, Saleh S, Parhar RS, Khabar K, Collison K (2005) Human neutrophil gene expression profiling following xenogeneic encounter with porcine aortic endothelial cells: the occult role of neutrophils in xenograft rejection revealed. J Leukoc Biol 78(1):51–61. https://doi.org/10.1189/jlb.0904494

Aron Badin R, Vadori M, Vanhove B, Nerriere-Daguin V, Naveilhan P, Neveu I, Jan C, Leveque X, Venturi E, Mermillod P, Van Camp N, Dolle F, Guillermier M, Denaro L, Manara R, Citton V, Simioni P, Zampieri P, D'Avella D, Rubello D, Fante F, Boldrin M, De Benedictis GM, Cavicchioli L, Sgarabotto D, Plebani M, Stefani AL, Brachet P, Blancho G, Soulillou JP, Hantraye P, Cozzi E (2016) Cell therapy for Parkinson's disease: a translational approach to assess the role of local and systemic immunosuppression. Am J Transplant 16(7):2016–2029. https://doi.org/10.1111/ajt.13704

Atkinson MA, Eisenbarth GS, Michels AW (2014) Type 1 diabetes. Lancet (London, England) 383 (9911):69–82. https://doi.org/10.1016/S0140-6736(13)60591-7

Bahr A, Kaser T, Kemter E, Gerner W, Kurome M, Baars W, Herbach N, Witter K, Wunsch A, Talker SC, Kessler B, Nagashima H, Saalmuller A, Schwinzer R, Wolf E, Klymiuk N (2016) Ubiquitous LEA29Y expression blocks T cell co-stimulation but permits sexual reproduction in genetically modified pigs. PLoS One 11(5):e0155676. https://doi.org/10.1371/journal.pone.0155676

Bakdash G, Sittig SP, van Dijk T, Figdor CG, de Vries IJ (2013) The nature of activatory and tolerogenic dendritic cell-derived signal II. Front Immunol 4:53. https://doi.org/10.3389/fimmu.2013.00053

Barkai U, Rotem A, de Vos P (2016) Survival of encapsulated islets: more than a membrane story. World J Transplant 6(1):69–90. https://doi.org/10.5500/wjt.v6.i1.69

Bartlett ST, Markmann JF, Johnson P, Korsgren O, Hering BJ, Scharp D, Kay TW, Bromberg J, Odorico JS, Weir GC, Bridges N, Kandaswamy R, Stock P, Friend P, Gotoh M, Cooper DK, Park CG, O'Connell P, Stabler C, Matsumoto S, Ludwig B, Choudhary P, Kovatchev B, Rickels MR, Sykes M, Wood K, Kraemer K, Hwa A, Stanley E, Ricordi C, Zimmerman M, Greenstein J, Montanya E, Otonkoski T (2016) Report from IPITA-TTS opinion leaders meeting on the future of beta-cell replacement. Transplantation 100(Suppl 2):S1–S44. https://doi.org/10.1097/tp.0000000000001055

Baumann BC, Stussi G, Huggel K, Rieben R, Seebach JD (2007) Reactivity of human natural antibodies to endothelial cells from Galalpha(1,3)Gal-deficient pigs. Transplantation 83(2):193–201. https://doi.org/10.1097/01.tp.0000250478.00567.e5

Bistoni G, Calvitti M, Mancuso F, Arato I, Falabella G, Cucchia R, Fallarino F, Becchetti A, Baroni T, Mazzitelli S, Nastruzzi C, Bodo M, Becchetti E, Cameron DF, Luca G, Calafiore R

(2012) Prolongation of skin allograft survival in rats by the transplantation of microencapsulated xenogeneic neonatal porcine Sertoli cells. Biomaterials 33(21):5333–5340. https://doi.org/10.1016/j.biomaterials.2012.04.020

Bottino R, Wijkstrom M, van der Windt DJ, Hara H, Ezzelarab M, Murase N, Bertera S, He J, Phelps C, Ayares D, Cooper DK, Trucco M (2014) Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. Am J Transplant 14(10):2275–2287. https://doi.org/10.1111/ajt.12868

Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ (2014) Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges. Diabetes Metab Syndr Obes 7:211–223. https://doi.org/10.2147/DMSO.S50789

Byrne GW, McGregor CG, Breimer ME (2015) Recent investigations into pig antigen and anti-pig antibody expression. Int J Surg (London, England) 23(Pt B):223–228. https://doi.org/10.1016/j.ijsu.2015.07.724

Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, Bello-Laborn H, Hacquoil B, Strobert E, Gangappa S, Weber CJ, Pearson TC, Rajotte RV, Larsen CP (2006) Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 12(3):304–306. https://doi.org/10.1038/nm1375

Carson JL, Grossman BJ, Kleinman S, Tinmouth AT, Marques MB, Fung MK, Holcomb JB, Illoh O, Kaplan LJ, Katz LM, Rao SV, Roback JD, Shander A, Tobian AA, Weinstein R, Swinton McLaughlin LG, Djulbegovic B (2012) Red blood cell transfusion: a clinical practice guideline from the AABB*. Ann Intern Med 157(1):49–58. https://doi.org/10.7326/0003-4819-157-1-201206190-00429

Chen L, Flies DB (2013) Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 13(4):227–242. https://doi.org/10.1038/nri3405

Chen X, Feng X, Xie J, Ruan S, Lin Y, Lin Z, Shen R, Zhang F (2013) Application of acellular dermal xenografts in full-thickness skin burns. Exp Ther Med 6(1):194–198. https://doi.org/10.3892/etm.2013.1114

Choi HJ, Lee JJ, Kim DH, Kim MK, Lee HJ, Ko AY, Kang HJ, Park C, Wee WR (2015) Blockade of CD40-CD154 costimulatory pathway promotes long-term survival of full-thickness porcine corneal grafts in nonhuman primates: clinically applicable xenocorneal transplantation. Am J Transplant 15(3):628–641. https://doi.org/10.1111/ajt.13057

Choudhary P, Rickels MR, Senior PA, Vantyghem MC, Maffi P, Kay TW, Keymeulen B, Inagaki N, Saudek F, Lehmann R, Hering BJ (2015) Evidence-informed clinical practice recommendations for treatment of type 1 diabetes complicated by problematic hypoglycemia. Diabetes Care 38(6):1016–1029. https://doi.org/10.2337/dc15-0090

Cohen D, Miyagawa Y, Mehra R, Lee W, Isse K, Long C, Ayares DL, Cooper DK, Hara H (2014) Distribution of non-gal antigens in pig cornea: relevance to corneal xenotransplantation. Cornea 33(4):390–397. https://doi.org/10.1097/ICO.0000000000000069

Cooper DK, Hara H, Yazer M (2010) Genetically engineered pigs as a source for clinical red blood cell transfusion. Clin Lab Med 30(2):365–380. https://doi.org/10.1016/j.cll.2010.02.001

Cooper DK, Satyananda V, Ekser B, van der Windt DJ, Hara H, Ezzelarab MB, Schuurman HJ (2014) Progress in pig-to-non-human primate transplantation models (1998–2013): a comprehensive review of the literature. Xenotransplantation 21(5):397–419. https://doi.org/10.1111/xen.12127

Cooper DK, Matsumoto S, Abalovich A, Itoh T, Mourad NI, Gianello PR, Wolf E, Cozzi E (2016a) Progress in clinical encapsulated islet xenotransplantation. Transplantation 100(11):2301–2308

Cooper DK, Ezzelarab MB, Hara H, Iwase H, Lee W, Wijkstrom M, Bottino R (2016b) The pathobiology of pig-to-primate xenotransplantation: a historical review. Xenotransplantation 23(2):83–105. https://doi.org/10.1111/xen.12219

Cooper DK, Ekser B, Ramsoondar J, Phelps C, Ayares D (2016c) The role of genetically engineered pigs in xenotransplantation research. J Pathol 238(2):288–299. https://doi.org/10.1002/path.4635

Cooper DK, Bottino R, Gianello P, Graham M, Hawthorne WJ, Kirk AD, Korsgren O, Park CG, Weber C (2016d) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes –

chapter 4: pre-clinical efficacy and complication data required to justify a clinical trial. Xenotransplantation 23(1):46–52. https://doi.org/10.1111/xen.12226

Costa C, Brokaw JL, Wang Y, Fodor WL (2003) Delayed rejection of porcine cartilage is averted by transgenic expression of alpha1,2-fucosyltransferase. FASEB J 7(1):109–111. https://doi.org/10.1096/fj.02-0630fje

Cowan PJ, Robson SC (2015) Progress towards overcoming coagulopathy and hemostatic dysfunction associated with xenotransplantation. Int J Surg (London, England) 23(Pt B):296–300. https://doi.org/10.1016/j.ijsu.2015.07.682

Cowan PJ, Ayares D, Wolf E, Cooper DK (2016) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 2b: genetically modified source pigs. Xenotransplantation 23(1):32–37. https://doi.org/10.1111/xen.12224

Cowley MJ, Weinberg A, Zammit NW, Walters SN, Hawthorne WJ, Loudovaris T, Thomas H, Kay T, Gunton JE, Alexander SI, Kaplan W, Chapman J, O'Connell PJ, Grey ST (2012) Human islets express a marked proinflammatory molecular signature prior to transplantation. Cell Transplant 21(9):2063–2078. https://doi.org/10.3727/096368911x627372

Cozzi E, Tonjes RR, Gianello P, Buhler LH, Rayat GR, Matsumoto S, Park CG, Kwon I, Wang W, O'Connell P, Jessamine S, Elliott RB, Kobayashi T, Hering BJ (2016) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 1: update on national regulatory frameworks pertinent to clinical islet xenotransplantation. Xenotransplantation 23(1):14–24. https://doi.org/10.1111/xen.12222

de Groot M, Schuurs TA, van Schilfgaarde R (2004) Causes of limited survival of microencapsulated pancreatic islet grafts. J Surg Res 121(1):141–150. https://doi.org/10.1016/j.jss.2004.02.018

Deng Z, Jin J, Zhao J, Xu H (2016) Cartilage defect treatments: with or without cells? Mesenchymal stem cells or chondrocytes? Traditional or matrix-assisted? A systematic review and meta-analyses. Stem Cells Int 2016:9201492. https://doi.org/10.1155/2016/9201492

Denner J, Mueller NJ (2015) Preventing transfer of infectious agents. Int J Surg (London, England) 23(Pt B):306–311. https://doi.org/10.1016/j.ijsu.2015.08.032

Denner J, Schuurman HJ, Patience C (2009) The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 5: strategies to prevent transmission of porcine endogenous retroviruses. Xenotransplantation 16(4):239–248. https://doi.org/10.1111/j.1399-3089.2009.00544.x

Denner J, Tonjes RR, Takeuchi Y, Fishman J, Scobie L (2016) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 5: recipient monitoring and response plan for preventing disease transmission. Xenotransplantation 23(1):53–59. https://doi.org/10.1111/xen.12227

Dhawan A, Puppi J, Hughes RD, Mitry RR (2010) Human hepatocyte transplantation: current experience and future challenges. Nat Rev Gastroenterol Hepatol 7(5):288–298. https://doi.org/10.1038/nrgastro.2010.44

Dufrane D, Gianello P (2012) Macro- or microencapsulation of pig islets to cure type 1 diabetes. World J Gastroenterol 18(47):6885–6893. https://doi.org/10.3748/wjg.v18.i47.6885

Dufrane D, Goebbels RM, Gianello P (2010) Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. Transplantation 90(10):1054–1062. https://doi.org/10.1097/TP.0b013e3181f6e267

Dunn TB, Kirchner V, Bellin MD (2015) Beta-cell replacement therapy: current outcomes and future landscape. Curr Opin Organ Transplant 20(6):681–690. https://doi.org/10.1097/MOT.0000000000000245

EC (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing

Council Directive 90/220/EEC. http://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A32001L0018

EC (2007) 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. http://ec.europa.eu/health/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf

Ekser B, Ezzelarab M, Hara H, van der Windt DJ, Wijkstrom M, Bottino R, Trucco M, Cooper DK (2012a) Clinical xenotransplantation: the next medical revolution? Lancet (London, England) 379(9816):672–683. https://doi.org/10.1016/S0140-6736(11)61091-X

Ekser B, Bianchi J, Ball S, Iwase H, Walters A, Ezzelarab M, Veroux M, Gridelli B, Wagner R, Ayares D, Cooper DK (2012b) Comparison of hematologic, biochemical, and coagulation parameters in alpha1,3-galactosyltransferase gene-knockout pigs, wild-type pigs, and four primate species. Xenotransplantation 19(6):342–354. https://doi.org/10.1111/xen.12007

Ekser B, Cooper DK, Tector AJ (2015a) The need for xenotransplantation as a source of organs and cells for clinical transplantation. Int J Surg (London, England) 23(Pt B):199–204. https://doi.org/10.1016/j.ijsu.2015.06.066

Ekser B, Markmann JF, Tector AJ (2015b) Current status of pig liver xenotransplantation. Int J Surg (London, England) 23(Pt B):240–246. https://doi.org/10.1016/j.ijsu.2015.06.083

Elliott RB, Escobar L, Tan PL, Garkavenko O, Calafiore R, Basta P, Vasconcellos AV, Emerich DF, Thanos C, Bambra C (2005) Intraperitoneal alginate-encapsulated neonatal porcine islets in a placebo-controlled study with 16 diabetic cynomolgus primates. Transplant Proc 37(8):3505–3508. https://doi.org/10.1016/j.transproceed.2005.09.038

Elliott RB, Escobar L, Tan PL, Muzina M, Zwain S, Buchanan C (2007) Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation. Xenotransplantation 14(2):157–161. https://doi.org/10.1111/j.1399-3089.2007.00384.x

Ellis C, Lyon JG, Korbutt GS (2016) Optimization and scale-up isolation and culture of neonatal porcine islets: potential for clinical application. Cell Transplant 25(3):539–547. https://doi.org/10.3727/096368915x689451

EMA (2009) Guideline on cell-based medicinal products. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/12/WC500016936.pdf

EMA (2012) Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/05/WC500126836.pdf

Emamaullee JA, Rajotte RV, Liston P, Korneluk RG, Lakey JR, Shapiro AM, Elliott JF (2005) XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes 54(9):2541–2548

Estrada JL, Martens G, Li P, Adams A, Newell KA, Ford ML, Butler JR, Sidner R, Tector M, Tector J (2015) Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. Xenotransplantation 22(3):194–202. https://doi.org/10.1111/xen.12161

Eventov-Friedman S, Tchorsh D, Katchman H, Shezen E, Aronovich A, Hecht G, Dekel B, Rechavi G, Blazar BR, Feine I, Tal O, Freud E, Reisner Y (2006) Embryonic pig pancreatic tissue transplantation for the treatment of diabetes. PLoS Med 3(7):e215. https://doi.org/10.1371/journal.pmed.0030215

Fallarino F, Luca G, Calvitti M, Mancuso F, Nastruzzi C, Fioretti MC, Grohmann U, Becchetti E, Burgevin A, Kratzer R, van Endert P, Boon L, Puccetti P, Calafiore R (2009) Therapy of experimental type 1 diabetes by isolated Sertoli cell xenografts alone. J Exp Med 206(11):2511–2526. https://doi.org/10.1084/jem.20090134

FDA (2016) Guidance for industry source animal, product, preclinical, and clinical issues concerning the use of xenotransplantation products in humans. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/xenotransplantation/ucm533036.pdf

FDA (2015) Guidance for industry, regulation of genetically engineered animals containing heritable recombinant DNA constructs. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm113903.pdf

FDA Cellular & Gene Therapy Products. http://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/

Fink JS, Schumacher JM, Ellias SL, Palmer EP, Saint-Hilaire M, Shannon K, Penn R, Starr P, VanHorne C, Kott HS, Dempsey PK, Fischman AJ, Raineri R, Manhart C, Dinsmore J, Isacson O (2000) Porcine xenografts in Parkinson's disease and Huntington's disease patients: preliminary results. Cell Transplant 9(2):273–278

Fischer K, Kraner-Scheiber S, Petersen B, Rieblinger B, Buermann A, Flisikowska T, Flisikowski K, Christan S, Edlinger M, Baars W, Kurome M, Zakhartchenko V, Kessler B, Plotzki E, Szczerbal I, Switonski M, Denner J, Wolf E, Schwinzer R, Niemann H, Kind A, Schnieke A (2016) Efficient production of multi-modified pigs for xenotransplantation by 'combineering', gene stacking and gene editing. Sci Rep 6:29081. https://doi.org/10.1038/srep29081

Fotino N, Fotino C, Pileggi A (2015) Re-engineering islet cell transplantation. Pharmacol Res 98:76–85. https://doi.org/10.1016/j.phrs.2015.02.010

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 U S A 97(25):13877–13882. https://doi.org/10.1073/pnas.97.25.13877

Gain P, Jullienne R, He Z, Aldossary M, Acquart S, Cognasse F, Thuret G (2016) Global survey of corneal transplantation and eye banking. JAMA Ophthalmol 134(2):167–173. https://doi.org/10.1001/jamaophthalmol.2015.4776

Gardoni F, Bellone C (2015) Modulation of the glutamatergic transmission by dopamine: a focus on Parkinson, Huntington and addiction diseases. Front Cell Neurosci 9:25. https://doi.org/10.3389/fncel.2015.00025

Gonzalez JG (2012) Ethical and regulatory issues for clinical trials in xenotransplantation. Methods Mol Biol 885:281–305. https://doi.org/10.1007/978-1-61779-845-0_18

Graham ML, Bellin MD, Papas KK, Hering BJ, Schuurman HJ (2011) Species incompatibilities in the pig-to-macaque islet xenotransplant model affect transplant outcome: a comparison with allotransplantation. Xenotransplantation 18(6):328–342. https://doi.org/10.1111/j.1399-3089.2011.00676.x

Griesemer A, Yamada K, Sykes M (2014) Xenotransplantation: immunological hurdles and progress toward tolerance. Immunol Rev 258(1):241–258. https://doi.org/10.1111/imr.12152

Gustafson EK, Elgue G, Hughes RD, Mitry RR, Sanchez J, Haglund U, Meurling S, Dhawan A, Korsgren O, Nilsson B (2011) The instant blood-mediated inflammatory reaction characterized in hepatocyte transplantation. Transplantation 91(6):632–638. https://doi.org/10.1097/TP.0b013e31820ae459

Hague SM, Klaffke S, Bandmann O (2005) Neurodegenerative disorders: Parkinson's disease and Huntington's disease. J Neurol Neurosurg Psychiatry 76(8):1058–1063. https://doi.org/10.1136/jnnp.2004.060186

Ham DS, Song MS, Park HS, Rhee M, Yang HK, Lee SH, Kim JW, Jung ES, Yoon KH (2015) Successful xenotransplantation with re-aggregated and encapsulated neonatal pig liver cells for treatment of mice with acute liver failure. Xenotransplantation 22(4):249–259. https://doi.org/10.1111/xen.12177

Hammerman MR (2013) Xenotransplantation of embryonic pig pancreas for treatment of diabetes mellitus in non-human primates. J Biomed Sci Eng 6(5a). https://doi.org/10.4236/jbise.2013.65A002

Hara H, Cooper DK (2010) The immunology of corneal xenotransplantation: a review of the literature. Xenotransplantation 17(5):338–349. https://doi.org/10.1111/j.1399-3089.2010.00608.x

Hara H, Cooper DK (2011) Xenotransplantation – the future of corneal transplantation? Cornea 30(4):371–378. https://doi.org/10.1097/ICO.0b013e3181f237ef

Hara H, Witt W, Crossley T, Long C, Isse K, Fan L, Phelps CJ, Ayares D, Cooper DK, Dai Y, Starzl TE (2013) Human dominant-negative class II transactivator transgenic pigs – effect on the

human anti-pig T-cell immune response and immune status. Immunology 140(1):39–46. https://doi.org/10.1111/imm.12107

Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, Lucas-Hahn A, Zhang L, Meng X, Gregory PD, Schwinzer R, Cost GJ, Niemann H (2011) Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proc Natl Acad Sci U S A 108(29):12013–12017. https://doi.org/10.1073/pnas.1106422108

Hawthorne WJ, Salvaris EJ, Phillips P, Hawkes J, Liuwantara D, Burns H, Barlow H, Stewart AB, Peirce SB, Hu M, Lew AM, Robson SC, Nottle MB, D'Apice AJ, O'Connell PJ, Cowan PJ (2014) Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. Am J Transplant 14(6):1300–1309. https://doi.org/10.1111/ajt.12722

Hecht G, Eventov-Friedman S, Rosen C, Shezen E, Tchorsh D, Aronovich A, Freud E, Golan H, El-Hasid R, Katchman H, Hering BJ, Zung A, Kra-Oz Z, Shaked-Mishan P, Yusim A, Shtabsky A, Idelevitch P, Tobar A, Harmelin A, Bachar-Lustig E, Reisner Y (2009) Embryonic pig pancreatic tissue for the treatment of diabetes in a nonhuman primate model. Proc Natl Acad Sci U S A 106(21):8659–8664. https://doi.org/10.1073/pnas.0812253106

Hering BJ, O'Connell PJ (2016) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 6: patient selection for pilot clinical trials of islet xenotransplantation. Xenotransplantation 23(1):60–76. https://doi.org/10.1111/xen.12228

Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, Ansite JD, Nakano M, Cheng J, Li W, Moran K, Christians U, Finnegan C, Mills CD, Sutherland DE, Bansal-Pakala P, Murtaugh MP, Kirchhof N, Schuurman HJ (2006) Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 12(3):301–303. https://doi.org/10.1038/nm1369

Hermans MH (2011) Preservation methods of allografts and their (lack of) influence on clinical results in partial thickness burns. Burns 37(5):873–881. https://doi.org/10.1016/j.burns.2011.01.007

Hughes RD, Mitry RR, Dhawan A (2012) Current status of hepatocyte transplantation. Transplantation 93(4):342–347. https://doi.org/10.1097/TP.0b013e31823b72d6

Iwase H, Kobayashi T (2015) Current status of pig kidney xenotransplantation. Int J Surg (London, England) 23(Pt B):229–233. https://doi.org/10.1016/j.ijsu.2015.07.721

Iwase H, Ekser B, Hara H, Phelps C, Ayares D, Cooper DK, Ezzelarab MB (2014) Regulation of human platelet aggregation by genetically modified pig endothelial cells and thrombin inhibition. Xenotransplantation 21(1):72–83. https://doi.org/10.1111/xen.12073

Ji H, Li X, Yue S, Li J, Chen H, Zhang Z, Ma B, Wang J, Pu M, Zhou L, Feng C, Wang D, Duan J, Pan D, Tao K, Dou K (2015) Pig BMSCs transfected with human TFPI combat species incompatibility and regulate the human TF pathway in vitro and in a rodent model. Cell Physiol Biochem 36(1):233–249. https://doi.org/10.1159/000374067

Jimenez-Vera E, Davies S, Phillips P, O'Connell PJ, Hawthorne WJ (2015) Long-term cultured neonatal islet cell clusters demonstrate better outcomes for reversal of diabetes: in vivo and molecular profiles. Xenotransplantation 22(2):114–123. https://doi.org/10.1111/xen.12151

Jiong C, Jiake C, Chunmao H, Yingen P, Qiuhe W, Zhouxi F, Xiangsheng F (2010) Clinical application and long-term follow-up study of porcine acellular dermal matrix combined with autoskin grafting. J Burn Care Res 31(2):280–285. https://doi.org/10.1097/BCR.0b013e3181d0f42d

Jitraruch S, Dhawan A, Hughes RD, Filippi C, Soong D, Philippeos C, Lehec SC, Heaton ND, Longhi MS, Mitry RR (2014) Alginate microencapsulated hepatocytes optimised for transplantation in acute liver failure. PLoS One 9(12):e113609. https://doi.org/10.1371/journal.pone.0113609

Karlsson-Parra A, Ridderstad A, Wallgren AC, Moller E, Ljunggren HG, Korsgren O (1996) Xenograft rejection of porcine islet-like cell clusters in normal and natural killer cell-depleted mice. Transplantation 61(9):1313–1320

Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB (2000) Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nat Med 6(2):114. https://doi.org/10.1038/72162

Kemter E, Lieke T, Kessler B, Kurome M, Wuensch A, Summerfield A, Ayares D, Nagashima H, Baars W, Schwinzer R, Wolf E (2012) Human TNF-related apoptosis-inducing ligand-expressing dendritic cells from transgenic pigs attenuate human xenogeneic T cell responses. Xenotransplantation 19(1):40–51. https://doi.org/10.1111/j.1399-3089.2011.00688.x

Kim MK, Hara H (2015) Current status of corneal xenotransplantation. Int J Surg (London, England) 23(Pt B):255–260. https://doi.org/10.1016/j.ijsu.2015.07.685

Kim MK, Wee WR, Park CG, Kim SJ (2011) Xenocorneal transplantation. Curr Opin Organ Transplant 16(2):231–236. https://doi.org/10.1097/MOT.0b013e328344870c

Kim MK, Choi HJ, Kwon I, Pierson RN 3rd, Cooper DK, Soulillou JP, O'Connell PJ, Vabres B, Maeda N, Hara H, Scobie L, Gianello P, Takeuchi Y, Yamada K, Hwang ES, Kim SJ, Park CG, International Xenotransplantation A (2014) The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of xenocorneal transplantation. Xenotransplantation 21(5):420–430. https://doi.org/10.1111/xen.12129

Kleine M, Schrem H, Borlak J, Klempnauer J (2008) Clinical versatility of porcine hepatocytes in the light of interspecies differences in cytochrome P450 regulation and expression. Xenotransplantation 15(4):208–217. https://doi.org/10.1111/j.1399-3089.2008.00478.x

Klose R, Kemter E, Bedke T, Bittmann I, Kelsser B, Endres R, Pfeffer K, Schwinzer R, Wolf E (2005) Expression of biologically active human TRAIL in transgenic pigs. Transplantation 80(2):222–230

Klymiuk N, Aigner B, Brem G, Wolf E (2010) Genetic modification of pigs as organ donors for xenotransplantation. Mol Reprod Dev 77(3):209–221. https://doi.org/10.1002/mrd.21127

Klymiuk N, van Buerck L, Bahr A, Offers M, Kessler B, Wuensch A, Kurome M, Thormann M, Lochner K, Nagashima H, Herbach N, Wanke R, Seissler J, Wolf E (2012a) Xenografted islet cell clusters from INSLEA29Y transgenic pigs rescue diabetes and prevent immune rejection in humanized mice. Diabetes 61(6):1527–1532. https://doi.org/10.2337/db11-1325

Klymiuk N, Bocker W, Schonitzer V, Bahr A, Radic T, Frohlich T, Wunsch A, Kessler B, Kurome M, Schilling E, Herbach N, Wanke R, Nagashima H, Mutschler W, Arnold GJ, Schwinzer R, Schieker M, Wolf E (2012b) First inducible transgene expression in porcine large animal models. FASEB J 26(3):1086–1099. https://doi.org/10.1096/fj.11-185041

Klymiuk N, Seeliger F, Bohlooly YM, Blutke A, Rudmann DG, Wolf E (2015) Tailored pig models for preclinical efficacy and safety testing of targeted therapies. Toxicol Pathol 44(3):346–357. https://doi.org/10.1177/0192623315609688

Klymiuk N, Ludwig B, Seissler J, Reichart B, Wolf E (2016) Current concepts of using pigs as a source for beta-cell replacement therapy of type 1 diabetes. Curr Mol Bio Rep 2(2):73–82. https://doi.org/10.1007/s40610-016-0039-1

Kobayashi T, Taniguchi S, Ye Y, Niekrasz M, Nour B, Cooper DK (1998) Comparison of bile chemistry between humans, baboons, and pigs: implications for clinical and experimental liver xenotransplantation. Lab Anim Sci 48(2):197–200

Korbutt GS (2008) What type of islets should be used? Xenotransplantation 15(2):81–82. https://doi.org/10.1111/j.1399-3089.2008.00455.x

Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV (1996) Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 97(9):2119–2129. https://doi.org/10.1172/jci118649

Korsgren O, Jansson L, Eizirik D, Andersson A (1991) Functional and morphological differentiation of fetal porcine islet-like cell clusters after transplantation into nude mice. Diabetologia 34(6):379–386

Kourtzelis I, Magnusson PU, Kotlabova K, Lambris JD, Chavakis T (2015) Regulation of instant blood mediated inflammatory reaction (IBMIR) in pancreatic islet xeno-transplantation: points for therapeutic interventions. Adv Exp Med Biol 865:171–188. https://doi.org/10.1007/978-3-319-18603-0_11

Kwon DN, Lee K, Kang MJ, Choi YJ, Park C, Whyte JJ, Brown AN, Kim JH, Samuel M, Mao J, Park KW, Murphy CN, Prather RS, Kim JH (2013) Production of biallelic CMP-Neu5Ac hydroxylase knock-out pigs. Sci Rep 3:1981. https://doi.org/10.1038/srep01981

Lai Y, Schneider D, Kidszun A, Hauck-Schmalenberger I, Breier G, Brandhorst D, Brandhorst H, Iken M, Brendel MD, Bretzel RG, Linn T (2005) Vascular endothelial growth factor increases functional beta-cell mass by improvement of angiogenesis of isolated human and murine pancreatic islets. Transplantation 79(11):1530–1536

Laird C, Burdorf L, Pierson RN 3rd (2016) Lung xenotransplantation: a review. Curr Opin Organ Transplant 21(3):272–278. https://doi.org/10.1097/MOT.0000000000000311

Lamm V, Hara H, Mammen A, Dhaliwal D, Cooper DK (2014) Corneal blindness and xenotransplantation. Xenotransplantation 21(2):99–114. https://doi.org/10.1111/xen.12082

Lee I, Wang L, Wells AD, Ye Q, Han R, Dorf ME, Kuziel WA, Rollins BJ, Chen L, Hancock WW (2003) Blocking the monocyte chemoattractant protein-1/CCR2 chemokine pathway induces permanent survival of islet allografts through a programmed death-1 ligand-1-dependent mechanism. J Immunol 171(12):6929–6935

Li J, Ezzelarab MB, Ayares D, Cooper DK (2014) The potential role of genetically-modified pig mesenchymal stromal cells in xenotransplantation. Stem Cell Rev 10(1):79–85. https://doi.org/10.1007/s12015-013-9478-8

Lin CC, Ezzelarab M, Hara H, Long C, Lin CW, Dorling A, Cooper DK (2010) Atorvastatin or transgenic expression of TFPI inhibits coagulation initiated by anti-nonGal IgG binding to porcine aortic endothelial cells. J Thromb Haemost 8(9):2001–2010. https://doi.org/10.1111/j.1538-7836.2010.03950.x

Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD et al (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science (New York, NY) 247(4942):574–577

Luca G, Calvitti M, Mancuso F, Falabella G, Arato I, Bellucci C, List EO, Bellezza E, Angeli G, Lilli C, Bodo M, Becchetti E, Kopchick JJ, Cameron DF, Baroni T, Calafiore R (2013) Reversal of experimental Laron syndrome by xenotransplantation of microencapsulated porcine Sertoli cells. J Control Release 165(1):75–81. https://doi.org/10.1016/j.jconrel.2012.08.028

Luca G, Cameron DF, Arato I, Mancuso F, Linden EH, Calvitti M, Falabella G, Szekeres K, Bodo M, Ricci G, Hansen BC, Calafiore R (2014) Xenograft of microencapsulated Sertoli cells for the cell therapy of type 2 diabetes mellitus in spontaneously diabetic nonhuman primates: preliminary data. Transplant Proc 46(6):1999–2001. https://doi.org/10.1016/j.transproceed.2014.06.053

Luca G, Mancuso F, Calvitti M, Arato I, Falabella G, Bufalari A, De Monte V, Tresoldi E, Nastruzzi C, Basta G, Fallarino F, Lilli C, Bellucci C, Baroni T, Aglietti MC, Giovagnoli S, Cameron DF, Bodo M, Calafiore R (2015) Long-term stability, functional competence, and safety of microencapsulated specific pathogen-free neonatal porcine Sertoli cells: a potential product for cell transplant therapy. Xenotransplantation 22(4):273–283. https://doi.org/10.1111/xen.12175

Ludwig B, Ludwig S (2015) Transplantable bioartificial pancreas devices: current status and future prospects. Langenbeck's Arch Surg 400(5):531–540. https://doi.org/10.1007/s00423-015-1314-y

Ludwig B, Rotem A, Schmid J, Weir GC, Colton CK, Brendel MD, Neufeld T, Block NL, Yavriyants K, Steffen A, Ludwig S, Chavakis T, Reichel A, Azarov D, Zimermann B, Maimon S, Balyura M, Rozenshtein T, Shabtay N, Vardi P, Bloch K, de Vos P, Schally AV, Bornstein SR, Barkai U (2012) Improvement of islet function in a bioartificial pancreas by enhanced oxygen supply and growth hormone releasing hormone agonist. Proc Natl Acad Sci U S A 109(13):5022–5027. https://doi.org/10.1073/pnas.1201868109

Ludwig B, Reichel A, Steffen A, Zimerman B, Schally AV, Block NL, Colton CK, Ludwig S, Kersting S, Bonifacio E, Solimena M, Gendler Z, Rotem A, Barkai U, Bornstein SR (2013) Transplantation of human islets without immunosuppression. Proc Natl Acad Sci U S A 110(47):19054–19058. https://doi.org/10.1073/pnas.1317561110

Ludwig B, Ludwig S, Steffen A, Zimermann B, Schmid J, Schubert U, Heinke S, Knauf Y, Kaup F-J, Goldman T, Barkai U, Rotem A, Bornstein S (2015) Preclinical studies on porcine islet macroencapsulation in non-human primates. Xenotransplantation 22(Suppl 1):S19–S20

Ludwig B, Ludwig S, Steffen A, Knauf Y, Zimerman B, Heinke S, Lehmann S, Schubert U, Schmid J, Bleyer M, Schönmann U, Colton CK, Bonifacio E, Solimena M, Reichel A, Schally AV, Rotem A, Barkai U, Grinberg-Rashi H, Kaup FJ, Avni Y, Jones P, Bornstein SR (2017) Favorable outcome of experimental islet xenotransplantation without immunosuppression in a nonhuman primate model of diabetes. Proc Natl Acad Sci U S A 114(44):11745–11750. https://doi.org/10.1073/pnas.1708420114

Lunney JK, Ho CS, Wysocki M, Smith DM (2009) Molecular genetics of the swine major histocompatibility complex, the SLA complex. Dev Comp Immunol 33(3):362–374. https://doi.org/10.1016/j.dci.2008.07.002

Lutz AJ, Li P, Estrada JL, Sidner RA, Chihara RK, Downey SM, Burlak C, Wang ZY, Reyes LM, Ivary B, Yin F, Blankenship RL, Paris LL, Tector AJ (2013) Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. Xenotransplantation 20(1):27–35. https://doi.org/10.1111/xen.12019

MacLeod TM, Sarathchandra P, Williams G, Sanders R, Green CJ (2004) Evaluation of a porcine origin acellular dermal matrix and small intestinal submucosa as dermal replacements in preventing secondary skin graft contraction. Burns 30(5):431–437. https://doi.org/10.1016/j.burns.2004.01.018

Mahou R, Passemard S, Carvello M, Petrelli A, Noverraz F, Gerber-Lemaire S, Wandrey C (2016) Contribution of polymeric materials to progress in xenotransplantation of microencapsulated cells: a review. Xenotransplantation 23(3):179–201. https://doi.org/10.1111/xen.12240

Martin BM, Samy KP, Lowe MC, Thompson PW, Cano J, Farris AB, Song M, Dove CR, Leopardi FV, Strobert EA, Jenkins JB, Collins BH, Larsen CP, Kirk AD (2015) Dual islet transplantation modeling of the instant blood-mediated inflammatory reaction. Am J Transplant 15(5):1241–1252. https://doi.org/10.1111/ajt.13098

Matsumoto S, Tomiya M, Sawamoto O (2016) Current status and future of clinical islet xenotransplantation. J Diabetes 8(4):483–493. https://doi.org/10.1111/1753-0407.12395

Meier RP, Navarro-Alvarez N, Morel P, Schuurman HJ, Strom S, Buhler LH (2015) Current status of hepatocyte xenotransplantation. Int J Surg (London, England) 23(Pt B):273–279. https://doi.org/10.1016/j.ijsu.2015.08.077

Mital P, Kaur G, Dufour JM (2010) Immunoprotective sertoli cells: making allogeneic and xenogeneic transplantation feasible. Reproduction (Cambridge, England) 139(3):495–504. https://doi.org/10.1530/rep-09-0384

Miyagawa S, Maeda A, Kawamura T, Ueno T, Usui N, Kondo S, Matsumoto S, Okitsu T, Goto M, Nagashima H (2014) A comparison of the main structures of N-glycans of porcine islets with those from humans. Glycobiology 24(2):125–138. https://doi.org/10.1093/glycob/cwt088

Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, Ostraat O, Salmela K, Tibell A, Tufveson G, Elgue G, Nilsson Ekdahl K, Korsgren O, Nilsson B (2002) Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet (London, England) 360(9350):2039–2045

Mohiuddin MM, Reichart B, Byrne GW, McGregor CG (2015) Current status of pig heart xenotransplantation. Int J Surg (London, England) 23(Pt B):234–239. https://doi.org/10.1016/j.ijsu.2015.08.038

Mohiuddin MM, Singh AK, Corcoran PC, Thomas ML 3rd, Clark T, Lewis BG, Hoyt RF, Eckhaus M, Pierson RN 3rd, Belli AJ, Wolf E, Klymiuk N, Phelps C, Reimann KA, Ayares D, Horvath KA (2016) Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. Nat Commun 7:11138. https://doi.org/10.1038/ncomms11138

Moll G, Jitschin R, von Bahr L, Rasmusson-Duprez I, Sundberg B, Lonnies L, Elgue G, Nilsson-Ekdahl K, Mougiakakos D, Lambris JD, Ringden O, Le Blanc K, Nilsson B (2011) Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. PLoS One 6(7):e21703. https://doi.org/10.1371/journal.pone.0021703

Mollon B, Kandel R, Chahal J, Theodoropoulos J (2013) The clinical status of cartilage tissue regeneration in humans. Osteoarthr Cartil 21(12):1824–1833. https://doi.org/10.1016/j.joca.2013.08.024

Morozumi K, Kobayashi T, Usami T, Oikawa T, Ohtsuka Y, Kato M, Takeuchi O, Koyama K, Matsuda H, Yokoyama I, Takagi H (1999) Significance of histochemical expression of Hanganutziu-Deicher antigens in pig, baboon and human tissues. Transplant Proc 31(1–2):942–944

Mourad N, Xhema D, Perota A, Galli C, Gianello P (2015) Beta-cell-specific expression of glucagon-like peptide 1 (GLP-1) and activated muscarinic receptor (M3R) improves pig islet secretory function. Xenotransplantation 22(Suppl. 1):S176

Mueller KR, Balamurugan AN, Cline GW, Pongratz RL, Hooper RL, Weegman BP, Kitzmann JP, Taylor MJ, Graham ML, Schuurman HJ, Papas KK (2013) Differences in glucose-stimulated insulin secretion in vitro of islets from human, nonhuman primate, and porcine origin. Xenotransplantation 20(2):75–81. https://doi.org/10.1111/xen.12022

Mulder A, Kardol MJ, Arn JS, Eijsink C, Franke ME, Schreuder GM, Haasnoot GW, Doxiadis II, Sachs DH, Smith DM, Claas FH (2010) Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. Mol Immunol 47(4):809–815. https://doi.org/10.1016/j.molimm.2009.10.004

Nagaraju S, Bottino R, Wijkstrom M, Trucco M, Cooper DK (2015) Islet xenotransplantation: what is the optimal age of the islet-source pig? Xenotransplantation 22(1):7–19. https://doi.org/10.1111/xen.12130

Nagata H, Nishitai R, Shirota C, Zhang JL, Koch CA, Cai J, Awwad M, Schuurman HJ, Christians U, Abe M, Baranowska-Kortylewicz J, Platt JL, Fox IJ (2007) Prolonged survival of porcine hepatocytes in cynomolgus monkeys. Gastroenterology 132(1):321–329. https://doi.org/10.1053/j.gastro.2006.10.013

Navarro-Alvarez N, Yang YG (2011) CD47: a new player in phagocytosis and xenograft rejection. Cell Mol Immunol 8(4):285–288. https://doi.org/10.1038/cmi.2010.83

Navarro-Alvarez N, Yang YG (2014) Lack of CD47 on donor hepatocytes promotes innate immune cell activation and graft loss: a potential barrier to hepatocyte xenotransplantation. Cell Transplant 23(3):345–354. https://doi.org/10.3727/096368913x663604

Neufeld T, Ludwig B, Barkai U, Weir GC, Colton CK, Evron Y, Balyura M, Yavriyants K, Zimermann B, Azarov D, Maimon S, Shabtay N, Rozenshtein T, Lorber D, Steffen A, Willenz U, Bloch K, Vardi P, Taube R, de Vos P, Lewis EC, Bornstein SR, Rotem A (2013) The efficacy of an immunoisolating membrane system for islet xenotransplantation in minipigs. PLoS One 8(8):e70150. https://doi.org/10.1371/journal.pone.0070150

Nilsson B, Ekdahl KN, Korsgren O (2011) Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. Curr Opin Organ Transplant 16(6):620–626. https://doi.org/10.1097/MOT.0b013e32834c2393

Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, Lesha E, Wang G, Luo Y, Qing Y, Jiao D, Zhao H, Zhou X, Wang S, Wei H, Güell M, Church GM, Yang L (2017) Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science 357(6357):1303–1307. https://doi.org/10.1126/science.aan4187

Oliva MS, Schottman T, Gulati M (2012) Turning the tide of corneal blindness. Indian J Ophthalmol 60(5):423–427. https://doi.org/10.4103/0301-4738.100540

Oropeza M, Petersen B, Carnwath JW, Lucas-Hahn A, Lemme E, Hassel P, Herrmann D, Barg-Kues B, Holler S, Queisser AL, Schwinzer R, Hinkel R, Kupatt C, Niemann H (2009) Transgenic expression of the human A20 gene in cloned pigs provides protection against apoptotic and inflammatory stimuli. Xenotransplantation 16(6):522–534. https://doi.org/10.1111/j.1399-3089.2009.00556.x

Park CG, Bottino R, Hawthorne WJ (2015) Current status of islet xenotransplantation. Int J Surg (London, England) 23(Pt B):261–266. https://doi.org/10.1016/j.ijsu.2015.07.703

Pepper AR, Gala-Lopez B, Pawlick R, Merani S, Kin T, Shapiro AMJ (2015) A prevascularized subcutaneous device-less site for islet and cellular transplantation. Nat Biotech 33(5):518–523. https://doi.org/10.1038/nbt.3211

Perkel JM (2016) Xenotransplantation makes a comeback. Nat Biotechnol 34(1):3–4. https://doi.org/10.1038/nbt0116-3

Petersen B, Ramackers W, Lucas-Hahn A, Lemme E, Hassel P, Queisser AL, Herrmann D, Barg-Kues B, Carnwath JW, Klose J, Tiede A, Friedrich L, Baars W, Schwinzer R, Winkler M,

Niemann H (2011) Transgenic expression of human heme oxygenase-1 in pigs confers resistance against xenograft rejection during ex vivo perfusion of porcine kidneys. Xenotransplantation 18(6):355–368. https://doi.org/10.1111/j.1399-3089.2011.00674.x

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL (2003) Production of alpha 1,3-galactosyltransferase-deficient pigs. Science (New York, NY) 299(5605):411–414. https://doi.org/10.1126/science.1078942

Plege A, Borns K, Baars W, Schwinzer R (2009) Suppression of human T-cell activation and expansion of regulatory T cells by pig cells overexpressing PD-ligands. Transplantation 87(7):975–982. https://doi.org/10.1097/TP.0b013e31819c85e8

Plege-Fleck A, Lieke T, Romermann D, Duvel H, Hundrieser J, Buermann A, Kraus L, Klempnauer J, Schwinzer R (2014) Pig to rat cell transplantation: reduced cellular and antibody responses to xenografts overexpressing PD-L1. Xenotransplantation 21(6):533–542. https://doi.org/10.1111/xen.12121

Qi M (2014) Transplantation of encapsulated pancreatic islets as a treatment for patients with type 1 diabetes mellitus. Adv Med 2014:429710. https://doi.org/10.1155/2014/429710

Ramackers W, Klose J, Vondran FW, Schrem H, Kaltenborn A, Klempnauer J, Kleine M (2014) Species-specific regulation of fibrinogen synthesis with implications for porcine hepatocyte xenotransplantation. Xenotransplantation 21(5):444–453. https://doi.org/10.1111/xen.12110

Rayat GR, Rajotte RV, Hering BJ, Binette TM, Korbutt GS (2003) In vitro and in vivo expression of Galalpha-(1,3)Gal on porcine islet cells is age dependent. J Endocrinol 177(1):127–135

Rayat GR, Gazda LS, Hawthorne WJ, Hering BJ, Hosking P, Matsumoto S, Rajotte RV (2016) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 3: porcine islet product manufacturing and release testing criteria. Xenotransplantation 23(1):38–45. https://doi.org/10.1111/xen.12225

Reichart B, Niemann H, Chavakis T, Denner J, Jaeckel E, Ludwig B, Marckmann G, Schnieke A, Schwinzer R, Seissler J, Tonjes RR, Klymiuk N, Wolf E, Bornstein SR (2015) Xenotransplantation of porcine islet cells as a potential option for the treatment of type 1 diabetes in the future. Horm Metab Res 47(1):31–35. https://doi.org/10.1055/s-0034-1395518

Reyes LM, Estrada JL, Wang ZY, Blosser RJ, Smith RF, Sidner RA, Paris LL, Blankenship RL, Ray CN, Miner AC, Tector M, Tector AJ (2014) Creating class I MHC-null pigs using guide RNA and the Cas9 endonuclease. J Immunol 193(11):5751–5757. https://doi.org/10.4049/jimmunol.1402059

Robles L, Storrs R, Lamb M, Alexander M, Lakey JR (2014) Current status of islet encapsulation. Cell Transplant 23(11):1321–1348. https://doi.org/10.3727/096368913X670949

Rogers SA, Chen F, Talcott MR, Faulkner C, Thomas JM, Thevis M, Hammerman MR (2007) Long-term engraftment following transplantation of pig pancreatic primordia into non-immunosuppressed diabetic rhesus macaques. Xenotransplantation 14(6):591–602. https://doi.org/10.1111/j.1399-3089.2007.00429.x

Rowan MP, Cancio LC, Elster EA, Burmeister DM, Rose LF, Natesan S, Chan RK, Christy RJ, Chung KK (2015) Burn wound healing and treatment: review and advancements. Crit Care 19:243. https://doi.org/10.1186/s13054-015-0961-2

Saudou F, Humbert S (2016) The biology of Huntingtin. Neuron 89(5):910–926. https://doi.org/10.1016/j.neuron.2016.02.003

Schrem H, Kleine M, Borlak J, Klempnauer J (2006) Physiological incompatibilities of porcine hepatocytes for clinical liver support. Liver Transpl 12(12):1832–1840. https://doi.org/10.1002/lt.20918

Schuurman HJ (2015) Regulatory aspects of clinical xenotransplantation. Int J Surg (London, England) 23(Pt B):312–321. https://doi.org/10.1016/j.ijsu.2015.09.051

Seol JG, Kim SH, Jin D, Hong SP, Yoo JY, Choi KM, Park YC, Yun YJ, Park KW, Heo JY (2010) Production of transgenic cloned miniature pigs with membrane-bound human Fas ligand (FasL)

by somatic cell nuclear transfer. Nature Precedings. https://precedings.nature.com/documents/4539/version/1

Shapiro AM (2012) Islet transplantation in type 1 diabetes: ongoing challenges, refined procedures, and long-term outcome. Rev Diabet Stud 9(4):385–406. https://doi.org/10.1900/rds.2012.9.385

Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355(13):1318–1330. https://doi.org/10.1056/NEJMoa061267

Shin JS, Kim JM, Kim JS, Min BH, Kim YH, Kim HJ, Jang JY, Yoon IH, Kang HJ, Kim J, Hwang ES, Lim DG, Lee WW, Ha J, Jung KC, Park SH, Kim SJ, Park CG (2015) Long-term control of diabetes in immunosuppressed nonhuman primates (NHP) by the transplantation of adult porcine islets. Am J Transplant 15(11):2837–2850. https://doi.org/10.1111/ajt.13345

Sommaggio R, Manez R, Costa C (2009) TNF, pig CD86, and VCAM-1 identified as potential targets for intervention in xenotransplantation of pig chondrocytes. Cell Transplant 18(12):1381–1393. https://doi.org/10.3727/096368909x474249

Sommaggio R, Perez-Cruz M, Brokaw JL, Manez R, Costa C (2013) Inhibition of complement component C5 protects porcine chondrocytes from xenogeneic rejection. Osteoarthr Cartil 21(12):1958–1967. https://doi.org/10.1016/j.joca.2013.09.002

Soucek P, Zuber R, Anzenbacherova E, Anzenbacher P, Guengerich FP (2001) Minipig cytochrome P450 3A, 2A and 2C enzymes have similar properties to human analogs. BMC Pharmacol 1:11

Speier S, Nyqvist D, Cabrera O, Yu J, Molano RD, Pileggi A, Moede T, Kohler M, Wilbertz J, Leibiger B, Ricordi C, Leibiger IB, Caicedo A, Berggren PO (2008a) Noninvasive in vivo imaging of pancreatic islet cell biology. Nat Med 14(5):574–578. https://doi.org/10.1038/nm1701

Speier S, Nyqvist D, Kohler M, Caicedo A, Leibiger IB, Berggren PO (2008b) Noninvasive high-resolution in vivo imaging of cell biology in the anterior chamber of the mouse eye. Nat Protoc 3(8):1278–1286. https://doi.org/10.1038/nprot.2008.118

Spizzo T, Denner J, Gazda L, Martin M, Nathu D, Scobie L, Takeuchi Y (2016) First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – chapter 2a: source pigs – preventing xenozoonoses. Xenotransplantation 23(1):25–31. https://doi.org/10.1111/xen.12223

Stephenne X, Vosters O, Najimi M, Beuneu C, Dung KN, Wijns W, Goldman M, Sokal EM (2007) Tissue factor-dependent procoagulant activity of isolated human hepatocytes: relevance to liver cell transplantation. Liver Transpl 13(4):599–606. https://doi.org/10.1002/lt.21128

Stewart ZA, Wilinska ME, Hartnell S, Temple RC, Rayman G, Stanley KP, Simmons D, Law GR, Scott EM, Hovorka R, Murphy HR (2016) Closed-loop insulin delivery during pregnancy in women with type 1 diabetes. N Engl J Med 375(7):644–654. https://doi.org/10.1056/NEJMoa1602494

Stone KR, Walgenbach AW, Abrams JT, Nelson J, Gillett N, Galili U (1997) Porcine and bovine cartilage transplants in cynomolgus monkey: I. A model for chronic xenograft rejection. Transplantation 63(5):640–645

Tena AA, Sachs DH, Mallard C, Yang YG, Tasaki M, Farkash E, Rosales IA, Colvin RB, Leonard DA, Hawley RJ (2016) Prolonged survival of pig skin on baboons after administration of pig cells expressing human CD47. Transplantation. https://doi.org/10.1097/tp.0000000000001267

Thompson P, Badell IR, Lowe M, Cano J, Song M, Leopardi F, Avila J, Ruhil R, Strobert E, Korbutt G, Rayat G, Rajotte R, Iwakoshi N, Larsen CP, Kirk AD (2011) Islet xenotransplantation using gal-deficient neonatal donors improves engraftment and function. Am J Transplant 11(12):2593–2602. https://doi.org/10.1111/j.1600-6143.2011.03720.x

Thompson P, Badell IR, Lowe M, Turner A, Cano J, Avila J, Azimzadeh A, Cheng X, Pierson RN 3rd, Johnson B, Robertson J, Song M, Leopardi F, Strobert E, Korbutt G, Rayat G, Rajotte R, Larsen CP, Kirk AD (2012) Alternative immunomodulatory strategies for xenotransplantation:

CD40/154 pathway-sparing regimens promote xenograft survival. Am J Transplant 12(7):1765–1775. https://doi.org/10.1111/j.1600-6143.2012.04031.x

Tomei AA, Manzoli V, Fraker CA, Giraldo J, Velluto D, Najjar M, Pileggi A, Molano RD, Ricordi C, Stabler CL, Hubbell JA (2014) Device design and materials optimization of conformal coating for islets of Langerhans. Proc Natl Acad Sci U S A 111(29):10514–10519. https://doi.org/10.1073/pnas.1402216111

Vabres B, Le Bas-Bernardet S, Riochet D, Cherel Y, Minault D, Hervouet J, Ducournau Y, Moreau A, Daguin V, Coulon F, Pallier A, Brouard S, Robson SC, Nottle MB, Cowan PJ, Venturi E, Mermillod P, Brachet P, Galli C, Lagutina I, Duchi R, Bach JM, Blancho G, Soulillou JP, Vanhove B (2014) hCTLA4-Ig transgene expression in keratocytes modulates rejection of corneal xenografts in a pig to non-human primate anterior lamellar keratoplasty model. Xenotransplantation 21(5):431–443. https://doi.org/10.1111/xen.12107

Vadori M, Cozzi E (2015) The immunological barriers to xenotransplantation. Tissue Antigens 86(4):239–253. https://doi.org/10.1111/tan.12669

Vadori M, Aron Badin R, Hantraye P, Cozzi E (2015) Current status of neuronal cell xenotransplantation. Int J Surg (London, England) 23(Pt B):267–272. https://doi.org/10.1016/j.ijsu.2015.09.052

Valdes-Gonzalez RA, Dorantes LM, Garibay GN, Bracho-Blanchet E, Mendez AJ, Davila-Perez R, Elliott RB, Teran L, White DJ (2005) Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. Eur J Endocrinol 153(3):419–427. https://doi.org/10.1530/eje.1.01982

Valdes-Gonzalez RA, White DJ, Dorantes LM, Teran L, Garibay-Nieto GN, Bracho-Blanchet E, Davila-Perez R, Evia-Viscarra L, Ormsby CE, Ayala-Sumuano JT, Silva-Torres ML, Ramirez-Gonzalez B (2007) Three-yr follow-up of a type 1 diabetes mellitus patient with an islet xenotransplant. Clin Transpl 21(3):352–357. https://doi.org/10.1111/j.1399-0012.2007.00648.x

van der Windt DJ, Bottino R, Casu A, Campanile N, Cooper DK (2007) Rapid loss of intraportally transplanted islets: an overview of pathophysiology and preventive strategies. Xenotransplantation 14(4):288–297. https://doi.org/10.1111/j.1399-3089.2007.00419.x

van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, Murase N, Hara H, Ball S, Loveland BE, Ayares D, Lakkis FG, Cooper DK, Trucco M (2009) Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. Am J Transplant 9(12):2716–2726. https://doi.org/10.1111/j.1600-6143.2009.02850.x

Vegas AJ, Veiseh O, Doloff JC, Ma M, Tam HH, Bratlie K, Li J, Bader AR, Langan E, Olejnik K, Fenton P, Kang JW, Hollister-Locke J, Bochenek MA, Chiu A, Siebert S, Tang K, Jhunjhunwala S, Aresta-Dasilva S, Dholakia N, Thakrar R, Vietti T, Chen M, Cohen J, Siniakowicz K, Qi M, McGarrigle J, Lyle S, Harlan DM, Greiner DL, Oberholzer J, Weir GC, Langer R, Anderson DG (2016a) Combinatorial hydrogel library enables identification of materials that mitigate the foreign body response in primates. Nat Biotechnol 34(3):345–352. https://doi.org/10.1038/nbt.3462

Vegas AJ, Veiseh O, Gurtler M, Millman JR, Pagliuca FW, Bader AR, Doloff JC, Li J, Chen M, Olejnik K, Tam HH, Jhunjhunwala S, Langan E, Aresta-Dasilva S, Gandham S, McGarrigle JJ, Bochenek MA, Hollister-Lock J, Oberholzer J, Greiner DL, Weir GC, Melton DA, Langer R, Anderson DG (2016b) Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 22(3):306–111. https://doi.org/10.1038/nm.4030

Veriter S, Gianello P, Igarashi Y, Beaurin G, Ghyselinck A, Aouassar N, Jordan B, Gallez B, Dufrane D (2014) Improvement of subcutaneous bioartificial pancreas vascularization and function by coencapsulation of pig islets and mesenchymal stem cells in primates. Cell Transplant 23(11):1349–1364. https://doi.org/10.3727/096368913x663550

Wang X, Meloche M, Verchere CB, Ou D, Mui A, Warnock GL (2011) Improving islet engraftment by gene therapy. J Transplant 2011:594851. https://doi.org/10.1155/2011/594851

Wang ZY, Burlak C, Estrada JL, Li P, Tector MF, Tector AJ (2014) Erythrocytes from GGTA1/CMAH knockout pigs: implications for xenotransfusion and testing in non-human primates. Xenotransplantation 21(4):376–384. https://doi.org/10.1111/xen.12106

Wang Y, Yang HQ, Jiang W, Fan NN, Zhao BT, Ou-Yang Z, Liu ZM, Zhao Y, Yang DS, Zhou XY, Shang HT, Wang LL, Xiang PY, Ge LP, Wei H, Lai LX (2015) Transgenic expression of human cytoxic T-lymphocyte associated antigen4-immunoglobulin (hCTLA4Ig) by porcine skin for xenogeneic skin grafting. Transgenic Res 24(2):199–211. https://doi.org/10.1007/s11248-014-9833-9

Weiner J, Yamada K, Ishikawa Y, Moran S, Etter J, Shimizu A, Smith RN, Sachs DH (2010) Prolonged survival of GalT-KO swine skin on baboons. Xenotransplantation 17(2):147–152. https://doi.org/10.1111/j.1399-3089.2010.00576.x

Weiss EH, Lilienfeld BG, Muller S, Muller E, Herbach N, Kessler B, Wanke R, Schwinzer R, Seebach JD, Wolf E, Brem G (2009) HLA-E/human beta2-microglobulin transgenic pigs: protection against xenogeneic human anti-pig natural killer cell cytotoxicity. Transplantation 87(1):35–43. https://doi.org/10.1097/TP.0b013e318191c784

Wheeler DG, Joseph ME, Mahamud SD, Aurand WL, Mohler PJ, Pompili VJ, Dwyer KM, Nottle MB, Harrison SJ, d'Apice AJ, Robson SC, Cowan PJ, Gumina RJ (2012) Transgenic swine: expression of human CD39 protects against myocardial injury. J Mol Cell Cardiol 52(5):958–961. https://doi.org/10.1016/j.yjmcc.2012.01.002

WHO Xenotransplantation. http://www.who.int/transplantation/xeno/en/

Wilson JT, Chaikof EL (2008) Thrombosis and inflammation in intraportal islet transplantation: a review of pathophysiology and emerging therapeutics. J Diabetes Sci Technol 2(5):746–759

Wolf-van Buerck L, Schuster M, Baehr A, Mayr T, Guethoff S, Abicht J, Reichart B, Nam-Apostolopoulos YC, Klymiuk N, Wolf E, Seissler J (2015) Engraftment and reversal of diabetes after intramuscular transplantation of neonatal porcine islet-like clusters. Xenotransplantation 22(6):443–450. https://doi.org/10.1111/xen.12201

Wolf-van Buerck L, Schuster M, Oduncu FS, Baehr A, Mayr T, Guethoff S, Abicht J, Reichart B, Klymiuk N, Wolf E, Seissler J (2017) LEA29Y expression in transgenic neonatal porcine islet-like cluster promotes long-lasting xenograft survival in humanized mice without immunosuppressive therapy. Sci Rep 7(1):3572. https://doi.org/10.1038/s41598-017-03913-4

Wright K, Dziuk R, Mital P, Kaur G, Dufour JM (2016) Xenotransplanted pig Sertoli cells inhibit both the alternative and classical pathways of complement mediated cell lysis while pig islets are killed. Cell Transplant. https://doi.org/10.3727/096368916x692032

Wuensch A, Baehr A, Bongoni AK, Kemter E, Blutke A, Baars W, Haertle S, Zakhartchenko V, Kurome M, Kessler B, Faber C, Abicht JM, Reichart B, Wanke R, Schwinzer R, Nagashima H, Rieben R, Ayares D, Wolf E, Klymiuk N (2014) Regulatory sequences of the porcine THBD gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multitransgenic pigs. Transplantation 97(2):138–147. https://doi.org/10.1097/TP.0b013e3182a95cbc

Wynyard S, Nathu D, Garkavenko O, Denner J, Elliott R (2014) Microbiological safety of the first clinical pig islet xenotransplantation trial in New Zealand. Xenotransplantation 21(4):309–323. https://doi.org/10.1111/xen.12102

Yang YG, Sykes M (2007) Xenotransplantation: current status and a perspective on the future. Nat Rev Immunol 7(7):519–531. https://doi.org/10.1038/nri2099

Yang HK, Yoon KH (2015) Current status of encapsulated islet transplantation. J Diabetes Complicat 29(5):737–743. https://doi.org/10.1016/j.jdiacomp.2015.03.017

Yang HK, Ham DS, Park HS, Rhee M, You YH, Kim MJ, Shin J, Kim OY, Khang G, Hong TH, Kim JW, Lee SH, Cho JH, Yoon KH (2015a) Long-term efficacy and biocompatibility of encapsulated islet transplantation with chitosan-coated alginate capsules in mice and canine models of diabetes. Transplantation 100(2):334–343. https://doi.org/10.1097/TP.0000000000000927

Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, Fishman JA, Church G (2015b) Genome-wide inactivation of

porcine endogenous retroviruses (PERVs). Science (New York, NY) 350(6264):1101–1104. https://doi.org/10.1126/science.aad1191

Zarrinpar A, Busuttil RW (2013) Liver transplantation: past, present and future. Nat Rev Gastroenterol Hepatol 10(7):434–440. https://doi.org/10.1038/nrgastro.2013.88

Zhang N, Richter A, Suriawinata J, Harbaran S, Altomonte J, Cong L, Zhang H, Song K, Meseck M, Bromberg J, Dong H (2004) Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 53(4):963–970

Zhang MC, Liu X, Jin Y, Jiang DL, Wei XS, Xie HT (2015) Lamellar keratoplasty treatment of fungal corneal ulcers with acellular porcine corneal stroma. Am J Transplant 15(4):1068–1075. https://doi.org/10.1111/ajt.13096

Zhou J, He W, Luo G, Wu J (2013) Fundamental immunology of skin transplantation and key strategies for tolerance induction. Arch Immunol Ther Exp (Warsz) 61(5):397–405. https://doi.org/10.1007/s00005-013-0233-2

Zhu HT, Lu L, Liu XY, Yu L, Lyu Y, Wang B (2015) Treatment of diabetes with encapsulated pig islets: an update on current developments. J Zhejiang Univ Sci B 16(5):329–343. https://doi.org/10.1631/jzus.B1400310

Part II

Cell Culture and Cell Modulation

Cell Culture Conditions: Cultivation of Stem Cells Under Dynamic Conditions

Andreas Clementi, Dominik Egger, Verena Charwat, and Cornelia Kasper

Contents

Abstract

A major challenge in tissue engineering and cell-therapy-based approaches is the production of a significant amount of functional cells. Although it does not represent a physiologic environment, most of stem cell cultivation is performed in static conditions (e.g., petri dishes). Furthermore, static expansion or differentiation of stem cells is labor-intensive and cells are often limited in number and functionality. In contrast, dynamic conditions (intentional active motion) enhance mass transfer and mechanotransductive effects which often results in higher numbers of functional cells. Specialized and partially automated bioreactor systems are widely used to transfer motion to cells and monitor important cultivation

A. Clementi · D. Egger · V. Charwat · C. Kasper (✉)
Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
e-mail: andreas.clementi@boku.ac.at; dominik.egger@boku.ac.at; verena.charwat@boku.ac.at; Cornelia.Kasper@boku.ac.at

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_58

parameters. Furthermore, in dynamic differentiation processes, bioreactors directly apply mechanical forces to generate physiologic conditions and enhance differentiation towards a specific lineage. Therefore, in this chapter, we discuss the application of dynamic conditions for the expansion and differentiation of stem cells. Consequently, a comprehensive overview of commercially available bioreactors for the expansion and differentiation of stem cells is presented.

1 Introduction

To date, most cell culture applications are performed in traditional dishes with cells grown on a plastic surface and growth medium applied on top of the cell layer. Except for minimal perturbation by Brownian motion, convection due to temperature or concentration gradients and (typically unintended) agitation as a consequence of handling, the liquid does not move in such cultures. Therefore, such systems are generally referred to as *static* cell cultures. In contrast *dynamic* cell cultivation is characterized by intentional active motion, which can be achieved directly by agitation of the culture medium (e.g., via pumps or impellers) or indirectly by agitation of the culture vessel (e.g., on a shaker platform). The earliest examples of dynamic animal cell cultures date back more than 80 years and were developed to eliminate drawbacks such as cumbersome handling associated with the commonly used cell culture methods of that time. In 1933, Gey developed the roller tube technique, which remains in use today (Gey 1933). Cells are grown adhered to the inner surface of a tube that is revolving at a defined slow speed. Other dynamic cell culture strategies have been adopted from related research fields. Most prominently, stirred-tank bioreactors have long been used for cultivation of microorganisms such as bacteria and yeast. In the 1970s and early 1980s, the adaptation of these systems for animal cell cultivation was developed. By that time the first glycosylated proteins for clinical trials were successfully expressed in animal cell lines. These achievements were a main driving force in the development of dynamic cell culture strategies since the industry was eager to develop safe, reproducible, and economically profitable production strategies (Jayapal et al. 2007). An early example constitutes the large-scale production of interferon in a human lymphoblastic cell line using a magnetic bar for stirring of the suspension culture (Reuveny et al. 1979). From the inception of such approaches, it was recognized that animal cells are less resilient to fluid dynamic forces compared to microorganisms due to their relatively large size and lack of a cell wall. Over time different strategies to address the hydrodynamic issues of dynamic animal cell cultivation were implemented, and various types of bioreactors specifically designed for animal cell cultivation were developed. For example, stirred tank and air lift bioreactors were modified to cultivate not only suspension cells but also adherently growing cell types on microcarriers (Croughan et al. 1987; Kunas and Papoutsakis 1990). Another approach is the physical separation of immobilized cells from fluid mechanical forces by membranes or porous walls (Tyler 1990; Vournakis and Runstadler

1990). Alternatively, specialized low-shear bioreactors have also been designed (Goodwin et al. 1993).

With the implementation of more gentle devices and protocols, dynamic cultivation could be applied to a number of different cell types and applications. As well as production cell lines, such as Chinese hamster ovary (CHO) and NS0 murine myeloma cells, which have been optimized for the secretion of recombinant proteins, primary cells are nowadays successfully cultivated using dynamic systems. In most cases, the goal is not protein production, but the cells themselves are the desired product. This also applies to large-scale cultivation of stem cells, which are intended/used in clinical applications. In stem cell therapies, stem cells are administered to treat or prevent a disease or condition. This, in the stem cell/cell therapy arena, is perhaps best exemplified by bone-marrow transplantation in cancer patients. Other relatively novel clinical applications include the treatment of graft-versus-host disease, immunological diseases, and adenosine deaminase deficiency. Besides the few approved stem cell therapies, many more clinical applications of stem cells are currently under investigation. A common characteristic of stem cell therapies is that typically large numbers of cells are required for each treatment (Trounson and McDonald 2015). For example, 10^7–10^8 mesenchymal stem cells (MSCs) are needed for one dose of immunosuppressive treatment after kidney transplantation (Reinders et al. 2013, 2014). Therefore, the cells cannot be used directly after isolation from healthy tissue of the patient (autologous) or a donor (allogeneic) but require a cell expansion step. Dynamic cell cultivation inside a bioreactor is usually performed to generate sufficient cell amounts from a small starting cell number. Compared to traditional cell cultivation strategies in static flasks, bioreactors provide some important advantages. The main benefits of dynamic cell cultivation technology include (i) improved process control; (ii) monitoring, documentation, and automation in computer-controlled systems; (iii) easier scale-up, reduction of handling, and manual labor; and (iv) improved reproducibility, reliability, and reduction of the required floor space and incubator volume for a given manufacturing goal.

While cell expansion systems seek to generate large numbers of undifferentiated stem cells for cell therapies, the targeted differentiation of stem cells is an important area of dynamic stem cell cultivation. Tissue engineering is based on the retrieval of cells from healthy donor tissue, followed by expansion and in vitro generation of a functional three-dimensional (3D) tissue graft that can then be used to replace or repair a defect in the patient's body. For generation and maturation of the tissue engineered construct, specialized bioreactors are often used. In contrast to two-dimensional cultures, the supply of nutrients and oxygen is a critical issue in 3D cell cultures. Dynamic cultivation strategies in combination with porous scaffolds or engineered vessel structures help to overcome diffusion limitation of supply. Therefore, dynamic cultivation is crucial to successfully establishing and maintaining any tissue engineered construct that is thicker than a few hundred micrometers. In addition, the mechanical stimulation of cells in a dynamic bioreactor is important. It is well known that stem cells are sensitive to mechanical cues, which have been shown to promote differentiation (Hao et al. 2015; Steward and Kelly 2015). While unwanted in cell expansion, mechanical stimulation of stem cells is intentionally

applied in tissue engineering bioreactors to induce or enhance targeted differentiation for example into osteogenic or chondrogenic lineage. Typically, the most successful stimulation with regard to mode of action (e.g., compression, tension, or shear stress), intensity and frequency is achieved by mimicking the conditions present in the respective tissue in vivo. Given the requirements for tissue engineering bioreactors vary significantly depending on the application, most systems are tailor-made, while only few commercial bioreactors exist.

Dynamic culture systems are also pivotal as in vitro test models, as well as in the clinical use of stem cells for cell therapy and tissue engineering. This includes the use of stem cells for drug development and testing, and toxicity tests. The requirements for in vitro test models are fundamentally different from clinical applications. While facing much lower regulatory demands, in vitro assays typically require high throughput and establishment of parallel systems, simple handling, high reproducibility, and fast and reliable analysis at reasonable cost. Consequently, cell-based testing is typically performed in miniaturized formats including 96- and 384-well plates or microarrays, which are designed for static cell cultivation. Dynamic stem cell models are often established in microfluidic devices or tailor-made research systems, while only few commercial platforms are available.

In this chapter, an overview of different approaches for dynamic cultivation of stem cells is provided. Since embryonic stem cells are ethically questionable (Hyun 2010), and induced pluripotent stem cells are still not considered as genetically stable (Laurent et al. 2011). This chapter concentrates on the cultivation of adult stem cells which are still the most widely used kind of stem cells. The cultivation systems are subdivided into bioreactors for expansion and differentiation based on their primary field of application. However, it should be noted that some bioreactors can be used in different configuration for expansion as well as differentiation. For each type of bioreactor, the underlying principle, mode of operation, brief historical facts, and advantages and limitations for stem cell cultivation are highlighted. Additionally, some important commercial systems are presented to exemplify the different concepts of dynamic stem cell cultivation. Although it is important to realize, it is not intended or indeed practical, to provide a comprehensive list of all available dynamic culture systems.

2 Dynamic Cultivation for Expansion of Stem Cells

An important requirement for the future success of regenerative medicine will be the availability of scalable stem cell expansion technologies. At the time of writing, research is currently still widely performed in planar vessels, which require cumbersome handling procedures, are not compatible with regulatory requirements, pose problems for downstream processing, and are difficult to scale up. These disadvantages largely contribute to the immense translational challenges currently faced in the field, where a vast number of research studies and preclinical testing produce only very few new approved stem cell treatments. Dynamic stem cell expansion in bioreactors seeks to overcome these limitations (Schnitzler et al. 2016).

Based on the general setup and configuration, the systems can be divided into four main categories: (i) wave-mixed, (ii) stirred, (iii) rotating bed and, and (iv) perfusion bioreactors. The main difference between these concepts is associated with mass transport (e.g., diffusion, perfusion, or bubbling), fluid mechanical forces (enhanced mass transfer at low shear stresses), and the ability to support three-dimensional culture constructs. Other distinctive features include scalability and available volumes, the degree of sensor integration and automation, and disposable versus reusable systems.

The process of cell expansion always follows the same general steps with variations dependent on the respective bioreactor system. First, the cells that need to proliferate are seeded into the bioreactor either directly or with a static pre-incubation step. Since stem cells are anchorage dependent, usually a substrate for cell adhesion is provided. The available formats include 2D surfaces (wall of roller flasks, stacked cell carrier slides), microcarriers, hollow fibers, and scaffolds with large interconnected pores. After an initial static incubation phase for cell attachment, the dynamic cultivation process is started. Movement of the culture medium ensures mixing, oxygen intake, and nutrient distribution. Depending on the integrated sensors and control units, the achievable degree of automation varies greatly between the different bioreactor systems. Most bioreactors are equipped with sensors for temperature, pH, CO_2, and oxygen, while samples need to be collected for external analysis of other parameters such as nutrient and metabolite levels (e.g., glucose consumption, lactate production) and cell number. Expansion processes can be set up as batch, fed-batch, continuous, and perfusion culture depending on the bioreactor type and application. Some expansion protocols also include cell passaging steps where the adherently growing stem cells are enzymatically detached and diluted to allow further expansion. Once expansion is established, a number of parameters need to be assessed to evaluate the success of a stem cell expansion protocol (Schnitzler et al. 2016): Obviously cell yield, growth rate, and viability are important criteria. However, besides achieving high cell numbers also the quality and "stemness" of the expanded cells has to be confirmed. Therefore, the correct expression of stem cell markers as well as the differentiation potential always needs to be evaluated. Other assays that are often performed include functional testing (e.g., immunomodulatory capacity and angiogenic potency), cell stress, and senescence assays and tests for tumorgenicity.

2.1 Wave-Mixed Bioreactors

The simplest form of dynamic cell cultivation is suspension culture vessels placed on a shaker platform. Compared to stirred systems, the main advantage is the noninvasive agitation which reduces the risk of contamination and facilitates easy handling and cleaning. The simplest basic setup consists of tubes or vials with a sterile filter included in the lid for air exchange that are placed on a shaker inside a cell culture incubator. These systems can be easily implemented in any cell culture laboratory with low cost and no need for additional infrastructure. However, the main limitation

of this crude type of bioreactor is the lack of automation and sensor integration. As a direct consequence, shaking flask and tube spin bioreactors are mainly used for stem cell culture in academic and research settings but clearly are not suitable for clinical grade production due to insufficient in-process control. However, a few advanced versions of orbitally shaken, small volume bioreactors are already on the market. One example is the Pall Micro-24 MicroReactor System, which allows 24 parallel experiments in 3–7 ml format with individual heating, gas injection, and pH and oxygen sensors. Another problem for stem cell expansion is that the agitation patterns of culture flasks on shaker platforms are typically far from ideal. In order to achieve good mixing and oxygen intake, relatively high rotation speeds are required, which may cause cell damage due to fluid mechanical shear stress. In order to overcome the issues of automation, process control, and efficient mixing, wave-mixed bag cultures have been developed. The most widely used and commercially available systems are the WAVE Bioreactors from GE Healthcare. A schematic concept is depicted in Fig. 1. A first version of the system was first introduced to the market in the 1990s as a disposable alternative to stainless steel bioreactors. It is operated outside of an incubator and consists of a single-use plastic bag filled with culture medium and cell inoculum, placed on a motorized platform. The platform performs a rocking motion, which provides excellent mixing and gas transfer without the effects of damaging fluid shear or gas bubbles (Singh 1999). Furthermore, the system is equipped with integrated sensor technology and control software

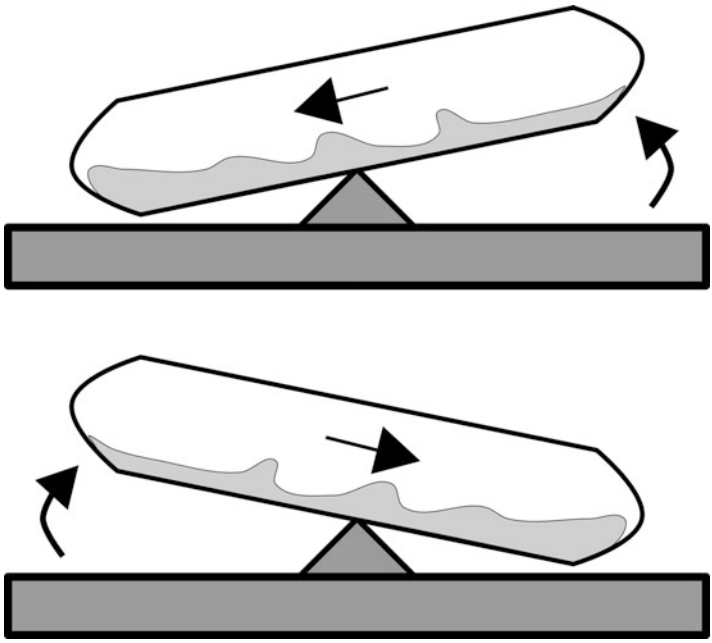


Fig. 1 Schematic concept of a wave-mixed bioreactor consisting of a disposable bag for suspension or microcarrier culture mounted on a rocking platform

to improve automation, reliability, and reproducibility. Today different versions and scales of the WAVE bioreactor family are available to resolve the needs of both research and GMP manufacturing applications (Somerville et al. 2012). Working volumes range from 300 ml to 500 l. However, while the system is well suited for medium to large-scale applications that require a high degree of automation, it is less suitable within academic settings where the high cost for bags and consumables as well as the low throughput and limited parallelization pose problems. Due to the continuous gas flow present in the system, the evaporation of cell culture medium becomes critical in small volumes (<500 ml). Integrated pH and dissolved oxygen (DO) sensors are only available for bags of 1 l and higher.

Importantly, the shaker-based cell culture systems have originally been developed for suspension cultures. While this mode of operation can be employed for microorganisms and some cell lines (e.g., CHO), stem cells typically cannot be maintained in suspension cultures. An exception was demonstrated by Mohamet et al. who used an E-cadherin blocking antibody in their mouse embryonic stem cell (ESC) cultures. The dispersed suspension culture was maintained as fed-batch culture in a shake-flask bioreactor for 16 days. A cell expansion of 2,775-fold was reported, while mESCs retained their pluripotency markers, high viability, and three germ layer differentiation potential (Mohamet et al. 2010). However, blocking of E-cadherin is expensive and inefficient for large-scale cultures, cannot be translated directly to human stem cells, and requires profound cell manipulation that is unwanted in most applications. For human (embryonic or induced) pluripotent stem cells (hPSCs), a similar protocol for expansion in a dynamic suspension culture has also been developed (Amit et al. 2011). A serum-free medium supplemented with interleukins and basic fibroblast growth factor was used to maintain undifferentiated hPSC spheres for 20 passages in Erlenmeyer flasks on an orbital shaker or in spinner flasks. These examples show that suspension cultivation of stem cells is possible under special circumstance. However, a much more common approach to culture stem cells and other adherently growing cell types in suspension bioreactors is the use of microcarriers.

Microcarriers were first introduced in 1967 as a biotechnological innovation to enable large-scale production of diploid and primary cells (Van Wezel 1967). Microcarriers allow the use of suspension bioreactors for adherently growing cells and facilitate process scale-up. Compared to culture flasks, microcarriers provide larger surface to volume ratios resulting in higher achievable cell culture densities. The medium agitation in the dynamic bioreactor does not only prevent sedimentation of the microcarriers but also ensures enhanced mass transfer to provide nutrients and oxygen to the cells, which can present limitations in static cultures. Microcarriers are small beads typically 125–250 μm diameter that can be maintained in suspension by relatively mild mixing and allow cell growth on the bead surface. While most microcarriers are spherical, other shapes such as cylindrical and disc-shaped also exist. Today a large number of different microcarriers are available: They are fabricated from many different materials such as dextran, polystyrene, collagen, alginate, and glass with macroporous or microporous inner structure. Surface properties are adjusted by physical or chemical modification. For stem cell

expansion, collagen or other compounds of the extracellular matrix (ECM) are commonly used as surface coating agent. More recently, also synthetic surfaces for cultivation of human MSCs in serum-free media have been developed (Dolley-Sonneville et al. 2013). While microcarriers provide a relatively easy and robust means of stem cell cultivation in suspension bioreactors, a number of limitations and disadvantages need to be considered. For example, it has been shown in multiple investigations that surface properties such as curvature, stiffness, and nano-topography influence stem cell behavior and may unintendedly induce differentiation (Zhao et al. 2014). Generally, softer materials tend to support the maintenance of stemness, while stiffer materials promote osteogenesis in human MSC (Engler et al. 2006). Another important consideration for cell expansion in microcarrier cultures is the efficiency of cell transfer from one bead to another, which depends on the cell type and properties of the microcarrier (e.g., surface coating) but is also influenced by medium composition (e.g., serum-based or serum-free) and agitation of the system (Hervy et al. 2014). In set-ups that do not promote efficient "inter-bead" cell migration, enzymatic treatment steps can be necessary to detach cells and populate fresh microcarriers. In some cases also complications like clumping and low cell recovery after harvest have been described as limiting factors in microcarrier-based cell cultures (Timmins et al. 2012)

Successful examples of shaker-mixed stem cell cultures include simple microcarrier-based systems in tube spin bioreactors and shaking flasks. Due to small vessel size and limited control, these systems are mainly used for pretesting or precultivation steps (Oh et al. 2009; Dove 2013). The potential of the WAVE system for stem cell expansion has been investigated (Jossen et al. 2014). Akerström et al. achieved a sixfold expansion of MSC within 18 days (Åkerström 2009). Similarly, human placental MSC have been cultivated and expanded in the WAVE system. A feasibility study using CultiSpher-S microcarriers suggested the potential to generate 7000 doses of 5 Mio cells/kg over four passages from a single placenta (Timmins et al. 2012).

2.2 Stirred Bioreactors

The stirred system is probably the oldest and the most classical reactor concept in biotechnology. These systems consist of a vessel and an impeller (see Fig. 2a) which is used to mix the culture medium in order to provide a homogenous environment regarding nutrient supply, oxygen concentration, cell concentration, and temperature. Due to this homogeneous environment, stirred systems are superior to most static or perfusion systems in terms of sampling, data collection, on-line monitoring, and control (Kumar et al. 2004). Additionally, stirred bioreactors provide a high degree of flexibility for increasing mixing and mass transfer by increasing stirrer speed and gassing rate. Engineering key parameters for stirred tank bioreactors are the rotations speed (rpm), tip speed (m s^{-1}), specific power input (W m^{-3}), Newton number (−), mixing time 95% (s), and volumetric oxygen transfer coefficient (s^{-1}). A common approach for engineers is the use of dimensionless numbers for concise

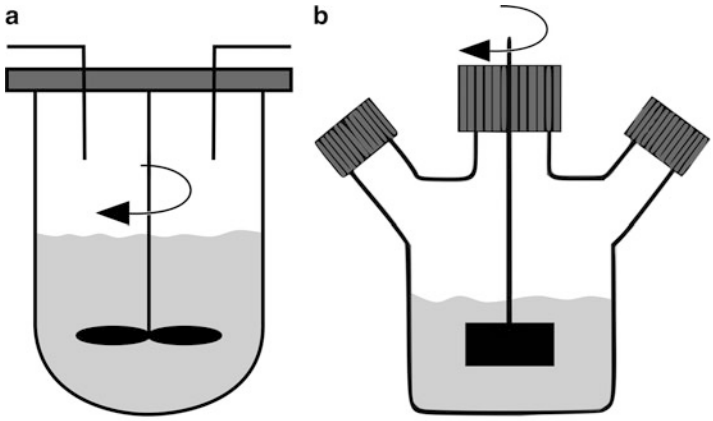


Fig. 2 (**a**) Schematic concept of a stirred tank bioreactor (**b**) Schematic concept of a spinner flask consisting of a main transparent body, two side openings, a main opening, and a stirrer

representation of physical phenomena. For example, a frequently used dimensionless number for the characterization of a stirred fluid is the Newton number N_P (also known as power number), which describes the power input from the impeller to the fluid:

$$N_P = \frac{P}{\rho N_i^3 D_i^5} \tag{1}$$

where N_i is rotation speed, D_i is impeller diameter, ρ is fluid density, μ is fluid viscosity, and P is power. Stirred tank reactors were first used in chemical engineering for a variety of applications. In cell culture technologies, adherent cell lines may be used, which brings an additional challenge, given adherent cells require a surface to adhere upon which complicates large-scale production in suspension culture (Glacken et al. 1983). This issue was overcome by cultivating cells on microcarriers or as spheroid suspension cultures (Alimperti et al. 2014). Such systems offer the same advantages as suspension cultures including high surface-to-volume ratio and reduced labor, material, and medium requirements (Glacken et al. 1983). Therefore, stirred-tank reactors have become the technology of choice for many large-scale cell culture applications, with the issues of adaptation to suspension culture, shear sensitivity, and oxygen supply mostly resolved (Chu and Robinson 2001). In contrast, the reactor technology for low volume and scientific applications remains diversified. Following the development of novel and commercially available stirred systems, a trend towards single-use technology has been observed. This trend responds to demands such as higher flexibility and responsiveness of production facilities and the reduction of manufacturing costs and timelines, in the context of increasingly strict regulatory and capacity requirements (Lopes 2015).

One promising candidate is the Mobius® product family. The system is a scalable platform of stirred tank bioreactors that provide flexibility by configuring software, hardware, and single-use assemblies for use in suspension and adherent cell culture applications, ranging from 3 to 2000 l in volume. The *Mobius 3L* bioreactor was introduced in 2009 and represents as a single use bioreactor between laboratory and pilot scale. This unbaffled bioreactor consists of a rigid plastic cultivation vessel with a total volume of 3 l, in which mixing is achieved by a marine impeller. The impeller's diameter is equal to 1/2 the tank diameter offering an off-bottom clearance of 0.028 m. The vessel has a diameter of 0.137 m with an H/D ratio of 1.82, and for aeration, a microsparger (sintered polyethylene, 15–30 mm pore size) is installed below the impeller (Kaiser et al. 2011). The *Mobius 3L* does not have built in sensors and motor, instead compatibility with various process control units is granted (Cierpka et al. 2013). For instance, a control unit by ez-Control (Applikon Biotechnology) was already used successfully for measuring pH, DO, and temperature in a CHO suspension culture (Eibl et al. 2010).

Widely used stirred systems for low volume applications are spinner-flask bioreactors. The main body consists of borosilicate glass (multiuse) or polystyrene (single-use), offering two smaller side openings with screw cap closures and one main central port for the stirrer (see Fig. 2b).

The side opening can be used for various tasks such as inoculation, medium inlet and outlet, sampling, pH probe inlet, and gassing with mixtures of O_2, CO_2, or N_2. Thus, spinner-flasks are versatile and can be used for different applications. Spinner flasks are available in sizes ranging from 125 ml up to 36 l in volume. Advantages of spinner flasks over traditional culture flasks are the flexibility for scale-up, reduced labor intensity, and their compact design. Compared to other stirred systems, spinner flasks are economical and are produced by a number of different companies offering a great variety of spinner flask types. For tissue engineering, the classical shape of the spinner flask was slightly modified to increase mixing and reduce shear force (Bilgen et al. 2006). This was achieved by changing the flask wall from a round to a wave-like shape which acts as baffles known from steel tank bioreactors. So-called wavy-walled bioreactors are suitable for cultivation of adherent cell types and frequently used for cell seeding and cultivation in 3D scaffolds.

2.3 Rotating Bed Bioreactors

Rotating bed bioreactors (RBB) are bioreactor systems where a fixed bed is slowly rotated through a liquid phase. The fixed bed is typically designed to provide a maximum surface-to-volume ratio as it serves as anchorage for adherent cells during cultivation. Gas control is often implemented through an oxygenator (gas permeable tubing with high surface-to-volume ratio) or the headspace of a half-filled culture vessel. In this case, the rotating bed is alternately rotated through the liquid medium for nutrition supply and the gas phase for oxygen supply. In contrast to stirred systems, which are associated with high fluid shear forces, RBBs are designed to enable proper mixing and mass transfer at low shear stress. Obviously, this is

advantageous for the cultivation of shear stress sensitive stem cells whose growth might be impaired due to impeller or carrier collisions in stirred bioreactors. Furthermore, cells can be harvested by enzymatic treatment and flushed out with the medium. For the purpose of expansion, it is less complicated to use a plain cell culture compatible plastic surface rather than rough or porous ones because cells trend to get trapped in pores or irregular shaped cavities. Still, for the purpose of differentiation of stem cells, other structured three-dimensional materials may serve as the fixed bed (Diederichs et al. 2009).

The Z®RP cell cultivation system of Zellwerk GmbH (Oberkrämer, Germany) is an RBB system that was mainly developed for the expansion of adherent cells. This RBB system consists of a breeder unit, a control unit, and the bioreactor itself. The breeder unit is comparable to a temperature controlled small scale sterile work bench with integrated UV-sterilization (Fig. 3). The control unit guarantees full control over any process. It is equipped with pumps for medium supply and base supply for pH control. Furthermore, the RBB system has a built-in gas mixing unit to generate any desired atmosphere including hypoxic conditions. The software allows to set all physical parameters (temperature, pO_2, pCO_2, pH) and medium exchange rates. The pH can be controlled via atmospheric CO_2 or titration of base. The bioreactor chamber is comparable to a magnetically driven rotating cell factory and hosts numerous layers of polycarbonate cell carrier slides. It is completely disposable while the motor allows to insert chambers of different size (2000–8000 cm^2 cell growth area). To account for varying needs of different cells, the bioreactor can be operated with different fluid levels and medium exchange rates. Slow rotation is applied to enhance mass transfer of oxygen and nutrients and to foster toxic waste removal. Critically, all components and the entire system can be operated according to GMP/GLP guidelines enabling application in a medical environment. Clearly, it is advantageous that the system remains closed during the entire cultivation period. Since medium exchange and physical parameters are automatically controlled, the risk of contamination is significantly reduced. Moreover, the programmed control ensures constant process outcomes including cell number and quality.

The system has been used for the expansion of umbilical cord derived MSC. Neumann et al. demonstrated an 8.2 ± 0.8 fold increase in cell number after a 5-day expansion. The cells were found to express immunophenotypic MSC surface markers and were able to differentiate into chondrogenic, osteogenic, and adipogenic lineage after expansion. Furthermore, no expression of β-galactosidase activity and, consequently, no cellular senescence was detected. In conclusion, bioreactor cultivation did not alter stemness or differentiation potential of the MSCs, important criteria for clinical application (Neumann et al. 2014). The system was also used for osteogenic differentiation of MSCs. For this, cells were seeded on porous ceramic 3D discs and cultivated for 47 days in osteogenic induction medium at a rotation of 1 rpm. Dynamically cultivated cells displayed a higher glucose consumption compared to statically cultivated cells. Moreover, matrix mineralization and alkaline phosphatase activity was increased compared to static conditions (Diederichs et al. 2009). The Z®RP rotating bed bioreactor appears to be a versatile system for stem

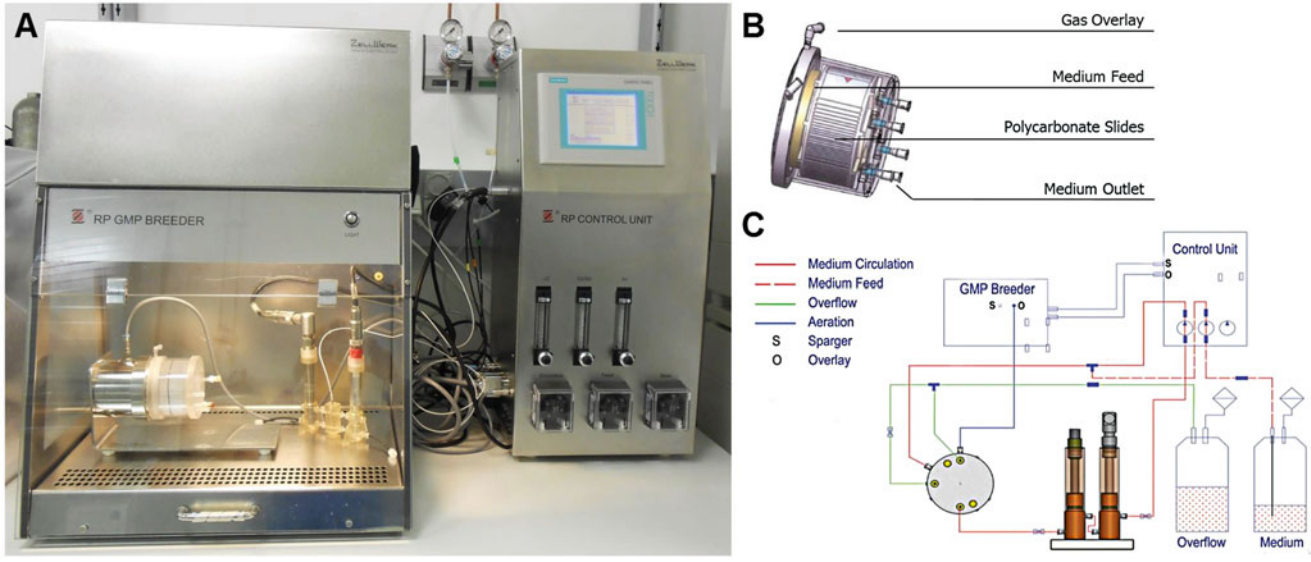


Fig. 3 (**a**) The ZRP system consists of the Breeder and the Control Unit. The rotating bed (**b**) is connected to a magnetic rotation engine. Sensors together with the Control Unit ensure comprehensive monitoring and control of the process (**c**)

cell expansion and differentiation with a high degree of automation that performs well over long cultivation periods.

The TisXell Regeneration System by QuinXell is not considered a traditional rotating bed bioreactor given the RBB allows the rotation around two axis. The rotation around the first axis causes a rotation of the "fixed bed" (i.e., the scaffold mount), while the rotation around the second axis causes the rotation of the spherical cultivation chamber wall. Therefore, the biaxial rotation makes this a versatile system with many possible applications. The cultivation chamber itself is connected to a medium reservoir and medium exchange is controlled via a peristaltic pump. A wide variety of chamber inserts are available to allow the use of various scaffolds, while chambers of different volumes (50,500 and 1000 ml) are available to host multiple scaffolds at once. The biaxial rotation enhances mixing and thus nutrient supply and waste removal. Rotation of both axis as well as the medium flow rate is controlled independently via a hand held control panel. The biaxial rotation introduces fluid shear forces to cells on the scaffold which are different to forces occurring in perfused systems. When cells grow confluent on the scaffold surface mass transfer to the core is limited, whereas in perfused systems the medium is forced to actively pass through the scaffold. The system is designed as a non-disposable bioreactor intended for use at research scale. It was first described by Zhang et al. (2009) and used for the osteogenic differentiation of human MSCs on polycaprolactone-tricalcium phosphate scaffolds. Notably, cell viability, cell number, ALP activity, and matrix mineralization were increased in the bioreactor compared to static cultivation. In comparison with a perfusion bioreactor, a spinner flask, and a rotating wall bioreactor, the biaxial rotating bioreactor displayed a higher cell yield and confluence as well as an earlier and more robust osteogenic differentiation (Zhang et al. 2010). Chondrogenic differentiation of MSCs on soft scaffolds was performed in the TisXell and increased matrix mineralization and chondrogenic gene expression were observed (Luciani et al. 2016). The system has been used for the testing of novel biomaterials for tissue engineering (Chhaya et al. 2015; Yassin et al. 2016).

In conclusion, both rotating bed bioreactors have successfully implemented dynamic conditions for stem cell expansion and differentiation, providing potential for such technology for further stem cell cultivation applications.

2.4 Hollow Fiber Bioreactors

In the field of cell and tissue culture technology, maintaining physiological and in vivo-like conditions during the cell culture process is a crucial task. Since it is know that cell layers beyond a thickness of 100 μm cannot be supported in vitro via diffusion under static conditions (Carrier et al. 2002), different culture systems have emerged in the recent years to overcome problems associated with 3D cell cultures. One approach is the application of hollow fiber bioreactors (HFB) which consist of fibers fixed into a module which offers a high surface area for cells to adhere and a continuous flow of media which is delivered through the fiber lumen. In Fig. 4a the

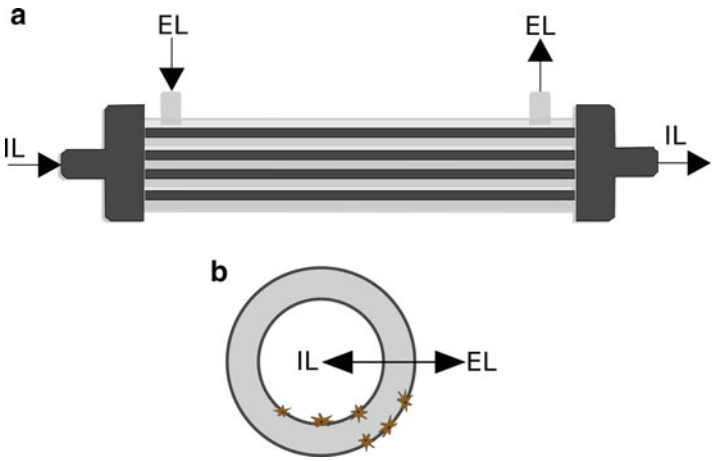


Fig. 4 (**a**) Concept of a hollow fiber bioreactor, showing in- and outlets for the intraluminal (IL) and extraluminal (EL) space. (**b**) Cross section of a fiber in the HFB. Cells can be seeded in the IL and/or EL space. Through the porous fiber wall O_2, CO_2, and nutrients are exchanged via diffusion

schematic concept of a HFB is illustrated. In principle, the bioreactor consists of two spaces: the intraluminal and the extraluminal space. Depending on the operational configuration, the flow of media (shown as arrows in Fig. 4a) can vary, e.g., co-current, counter-current, and dead-end configuration (Wung et al. 2014).

This setup offers a physiological environment with the fibers mimicking blood capillaries and shielding the cells from shear stress associated with dynamic media supply (Wung et al. 2014; Storm et al. 2016). Historically HFBs were first used in enzyme, microbial, and plant biotechnology. In 1983, the suitability of HFBs for mammalian cell cultures was mentioned (Vick Roy et al. 1983). HFBs were further optimized for adherent cell types and are known as versatile and modular systems regarding scale-up and the control of culture conditions. At the same time, HFBs offer high mass transport resulting in high cell densities (Wung et al. 2014; Eghbali et al. 2016). In Fig. 4b, a cross section of a fiber inside a HFB is shown. Cells can be seeded in the intraluminal and extraluminal space, which makes the HFB an interesting approach for cocultures. HFBs have been successfully used for culturing stromal and erythroleukemia cells as an in vitro bone marrow model for myeloid leukemia (Usuludin et al. 2012), performing a T-cell proliferation assay by culturing MSCs and peripheral blood mononuclear cells (Nold et al. 2013) and the cultivation of hepatocytes and nonparenchymal cells for a pharmacological in vitro model on human liver functions (Zeilinger et al. 2011).

In the studies mentioned above, most of the bioreactors were noncommercially available systems, prototypes, or self-made reactors. However, commercial HFB systems for cell expansion are also available, with the Quantum® Cell Expansion System (Terumo BCT) being the most advanced and widely used example. The Quantum® Cell Expansion System is a GMP compliant, closed cell culture platform for adherent cells that use hollow fibers to achieve a high surface-to-volume ratio.

The hollow fiber architecture used with the Quantum system contains approximately 11,500 single fibers, providing a 2.1 m^2 cell culture surface area, while occupying a total volume of only 180 ml. The same surface area can be achieved with 120 T-175 flasks, but requiring labor-intensive procedures with attendant risks of contamination. These challenges were resolved with the Quantum system by providing a high degree of automation which allows an adaptation to different cell culture tasks. Via a computer interface peristaltic pumps, fluid sensors, and tubing valves are controlled by the user. Additionally, harvested cells are automatically captured in a sterile 0.5 l bag that can be sealed for downstream processing. While this system allows to harvest $1.5 - 3 \times 10^8$ MSCs, it also needs a high amount ($\sim 2 \times 10^7$) of initial cells and medium (Rojewski et al. 2013; Lechanteur 2014). Notably, the Quantum system performs well for clinical applications, but due to the large size and cost its suitability for the research level is distinctly limited.

3 Dynamic Cultivation for Differentiation of Stem Cells

The differentiation of stem cells is an important aspect in tissue engineering. Although it is still of interest to engineer functional cell-matrix constructs for transplantation, there is also growing demand in functional tissue for dynamic 3D in vitro models. Therefore, dynamic conditions are not only important during differentiation of stem cells but also for maintaining physiologic conditions and thus functional tissue for in vitro test systems. In general, dynamic conditions are applied to generate and mimic a physiological environment that corresponds to the conditions cells would experience in their natural habitat. Adult stem cells are key players in tissue repair and regeneration. Whenever aged cells are damaged and need to be replaced, stem cells are recruited to the affected region where the stem cells start to differentiate into specific cell types. Every region in the body is subject to motion and therefore to mechanical forces. Although none of these forces can occur independently, the forces can be subdivided into three main categories: (i) compression, (ii) tension and, and (iii) shear forces (Fig. 5). Other forces like torsion, bending, or hydrostatic pressure are derived from these.

Mechanotransduction describes the translation of mechanical cues to biologic responses. The translation process is described as mechanical coupling, biochemical coupling, signal transmission, and effector response (Duncan and Turner 1995). Mechanical coupling is the physical transfer of mechanical forces to the biochemical sensors of a cell. These sensors are often mechanosensitive membrane proteins that change their conformation upon application of a force (biochemical coupling) which results in signal transmission by second messenger systems. Calcium transduction, G-protein, and kinase coupled signal transduction pathways are the most prominent systems that trigger the response at molecular level. Different mechanical forces activate various mechanosensitive receptors and thus lead to various effector responses. Compression has been mainly found to increase chondrogenesis of certain stem cells even in the absence of additional growth factors (Kisiday et al. 2009). It can be either applied directly to the cell-matrix construct or indirectly by

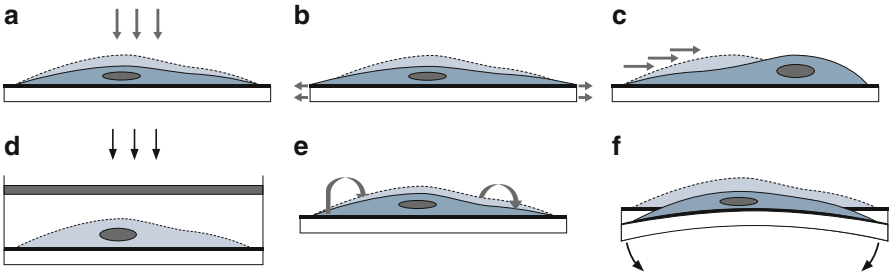


Fig. 5 Cells are subject to different mechanical forces in the human body. (**a**) Compression, (**b**) tension, and (**c**) shear are the three main forces, whereas (**d**) hydrostatic pressure, (**e**) torsion, and (**f**) bending are derived from these

Table 1 Characteristics of mechanical forces in the human body

Mechanical force	Induction of	Physiologic range	Reference
Compression	Chondrogenic differentiation	10–20 MPa	(Hodge et al. 1986)
Tension	Osteogenic, chondrogenic, ligament differentiation	15–30 MPa	(Noyes and Grood 1976; Johnson et al. 1994; Schechtman and Bader 1997)
Hydrostatic pressure	Chondrogenic differentiation	5–6 MPa average, up to 18 MPa peak loading	(Hodge et al. 1986)
Fluid shear stress	Osteogenic differentiation	0.8–3 Pa	(Weinbaum et al. 1994)

compression of the surrounding liquid which results in hydrostatic pressure. Tensile strain has been found to increase osteogenic (Rui et al. 2011), chondrogenic (McMahon et al. 2008), and ligamentous (Subramony et al. 2013) differentiation of stem cells. To apply tensile forces, cells are often seeded on a soft and flexible scaffold (e.g., collagen coated silicon) which are then placed into a special straining device. Shear forces are mostly applied by agitation of the surrounding fluid which can either overflow the construct or be passed through it. Fluid shear stress has been shown to increase osteogenesis, mainly (Arnsdorf et al. 2009).

The ultimate aim of bioreactors utilized in the differentiation of stem cells is the generation of mechanical forces within a defined physiologic range (Table 1). However, to generate and apply physiologic forces to a cell-matrix construct, many parameters need to be taken into account. Matrix characteristics like stiffness, average pore size, pore geometry, and overall porosity are crucial for preliminary calculations. Furthermore, fluid characteristics like dynamic and kinematic viscosity and density need to be considered. Finally, the bioreactor that applies the force needs to have an internal feedback loop to adjust mechanical forces during cultivation. For this, online monitoring of the forces or of parameters that are directly connected to them is crucial. Since the requirements of each process are different (and so are the

matrices, cells and fluids), it remains challenging to apply defined mechanical forces to a cell-matrix construct. However, until now only a few bioreactors have integrated sensors to measure and control the actual force that is applied to the cells.

Despite limited control and rudimentary understanding of the actual forces cells experienced, the first studies that implemented mechanical forces into their cell culture setup showed a striking impact on cell behavior. Not only cell viability and number were often increased but also ECM production, indicative of more functional tissue. Further studies confirmed the importance of dynamic cultivation conditions for the differentiation of stem cells. As a consequence, numerous bioreactors have been developed to apply compression, strain, fluid shear forces, or combinations of them on stem cells. The following sections explore the impact of the different mechanical forces and the concepts of how to apply these forces using bioreactor technology are detailed.

3.1 Compression

In mechanics, compression is the application of balanced inward (uniaxial) forces on two opposing points of a material which results in the reduction of its size in one or more directions. The phenomenon where a force (F) acts on an area (A) is called pressure (p) and is defined as $p = F/A$. Thus, for compression the SI unit Pascal ($Pa = kg\ m^{-1}\ s^{-2}$) or $N\ m^{-2}$ is used, but it can also be expressed in strain (ε, dimensionless, described in Sect. 3.2). In human biology, compression of tissue occurs naturally during everyday movements such as rising from a chair or walking down a stairway. These activities generate forces which are in the range of 10–20 MPa (Hodge et al. 1986) and usually affect bone and cartilage tissue. In the past, attention has focused on the ability of cells and tissues to respond to mechanical forces and other physical stimuli in their environment given it is known that mechanical forces can influence the fate of MSCs in vitro and in vivo. It has been demonstrated that forces which mimic physiological compression induce chondrogenic differentiation (Li et al. 2012). To understand this behavior on the molecular level, several studies have looked into the signaling pathways which are involved in mechanical stimulation of MSC differentiation. It was discovered that the ERK1/2 pathway determines whether MSCs differentiate toward osteogenic or chondrogenic lineage (Pelaez et al. 2012). Therefore, studies indicate that transduction of mechanical stimulation into chemical signals plays an important role in the differentiation of MSCs into a chondrogenic or osteogenic phenotype. Nevertheless, determination of the optimal frequency, load, and timing of mechanical stimulation on cultures for tissue engineering remains challenging. According to a recent review (Gaut and Sugaya 2015), the following approach is suggested:

1. Preconditioning MSCs with exogenous growth factors to increase their differentiation potential
2. Mechanical stimulation of differentiated MSCs for an optimal and natural ECM remodeling

3. The development of automated bioreactors that can provide a dynamic loading regime more similar to the native environment

One commercially available system for the application of compressive forces and other types of mechanical cues to cells is the CartiGen by BISS (Bangalore, India), which is also discussed with regards to the generation of hydrostatic pressure (see Sect. 3.3). For application of mechanical compression, the CartiGen bioreactor uses a linear motor with a displacement transducer, controlled and monitored by the GrowthWorks® software. The motor exerts a compressive force on a loading cell which offers different setups including:

C10-1: Consists of a single-sample static growth camber, dedicated for scaffolds of a maximum size range of 30 mm in diameter and 5 mm in thickness.
C10-12c: A multisample static growth chamber, dedicated for up to 12 disc shaped scaffolds with a maximum size of 10 mm in diameter and 5 mm in thickness. One advantage of this system is its optical grade window, which allows live cell imaging during cultivation.
C9-x: This system consists of a multisample perfusion chamber, allowing a steady perfusion of 9 disc shaped samples with a maximum size of 8 mm in diameter and 5 mm in thickness.

In one study, the CartiGen (C10-12c) bioreactor was used to apply dynamic long-term compression (3 h/day for 2 weeks) to primary human chondrocytes and thereby demonstrated an increased collagen type II and glycosaminoglycan expression (Jeon et al. 2012). Another group used the C9-x bioreactor for the cultivation of porcine chondrocytes in pellets, which were generated via centrifugation. The authors demonstrated that mechanically stimulated constructs exhibited an increase in total GAG content and different biomechanical properties compared to static constructs (Tran et al. 2011). Unfortunately, no data, at the time of writing, was available on the cultivation and differentiation of stem cells regarding the CartiGen product family. As stated in the introduction, the optimal approach to produce defined mechanical forces is via an internal feedback loop. This includes the use of integrated sensors which are able to constantly measure the applied force and give feedback signals to the actuator (e.g., motor or pump). Regarding the CartiGen bioreactor, a displacement transducer is used to monitor the position of the holding device, but the actual force applied to the construct is not measured.

3.2 Tension

Tension is the opposite of compression, describing the pulling forces applied axially on an object which results in an increase of size in one direction. Thus, the SI units Pascal (Pa) or N m^{-2} are used to describe tensile forces acting on a certain area. In material science, the unit strain (ε) is used to describe the fractional change in length while applying a certain tensile force. Strain is defined as:

$$\varepsilon = \frac{\Delta L}{L} \tag{2}$$

where ΔL is the change in length of an object while applying strain and L is the native length of an object. If the strain is negative, a compressive force is applied, and therefore, this principle can also be applied for compression. Tension or tensile strain in human physiology occurs, typically, in joints, tendons, bones, and muscles. For slow and concentric activities, the applied forces can be narrowed down to a range of 15–30 MPa (Noyes and Grood 1976; Johnson et al. 1994; Schechtman and Bader 1997) depending on many factors such as tissue, age, and weight. In the case of dynamic and impulsive activities, tensile forces experienced by tendons are in the range of 42–110 MPa which exceeds the established values of ultimate tensile strength (Johnson et al. 1994). Following the concept of mechanotransduction, the ability of stem cells to respond to mechanical tensile stimuli has also been assessed. It was shown that tensile forces play a significant role in regulating proliferation and differentiation pathways towards the osteogenic lineage (Kearney et al. 2010). Furthermore, tensile strain has also been found to influence the spatial rearrangement of human MSCs to form knob-like three-dimensional structures (Doyle et al. 2009) and the reorientation of HUVEC cells perpendicular to the axis of tension (Moretti et al. 2004). This suggests that mechanical cues may influence cell migration and orientation. On an intracellular level, as stated in Sect. 3.1 osteogenic differentiation is dependent on ERK1/2 pathway, but precise cellular mechanisms underlying the influence of mechanical signals to cellular responses, such as bone formation and regeneration, remain to be fully resolved. One interesting system for scientific purposes regarding tensile stimulation is the TC-3 bioreactor by Ebers Medical (Zaragoza, Spain) (Fig. 6). The bioreactor is designed to perform cell culture experiments with user-defined mechanical loading profiles such as tension and compression. Up to three chambers can be used in parallel by applying the same mechanical conditions. The system is therefore scalable and versatile since different mounting devices and a variety of scaffolds are available. The system allows cell culture chambers to be removed from the bioreactor for microscopy, avoiding unwanted interventions in the process such as the removal of the cell-matrix construct from the culture chamber. Control over the system is implemented by a simple computer interface which allows to define and store loading profiles.

Another system for the cultivation of adherent cell types which claims to apply tensile strains is the FX-5000™ Tension System from Flexcell® (Burlington, USA). This system does not use a motor to generate linear motion, instead a controlled vacuum is applied. Cells are seeded in monolayer on flexible bottomed culture plates which are then deformed by a vacuum on the outer layer. The culture plates are available in 24 well and 6 well formats and a maximal strain of 33% is achievable. The vacuum pump is controlled by the FX-5000™ software where the user can define cyclic or static strain. In one study, the FX-5000™ was used to investigate the effect of tensile strain to MSCs and fibroblasts in monolayer. The authors demonstrated that cell proliferation and ECM production is enhanced under tensile loading (Sun et al. 2016). The Flexcell® system is manly designed to study cellular and

Fig. 6 TC-3 bioreactor by Ebers Medical. (**a**) The system can be equipped with three independent chambers. Different chambers for the application of (**b**) hydrostatic pressure or (**c**) tension, compression and tension combined with compression are available. Pictures kindly provided by Ebers Medical

intracellular effects as a response to strain, rather than cultivate and differentiate stem cells in a controlled physiological environment.

In conclusion, current bioreactors on the market still lack the ability to perform mechanical cues in a physiological range for a potential translation of in vitro engineered tissue for clinical applications.

3.3 Hydrostatic Pressure

Hydrostatic pressure (HP) is a compressive force that is exerted by a stationary fluid in a closed system. It depends on the density of the fluid, the gravitational acceleration, and height of the liquid column above the given point. Thus, for incompressible fluids, HP can be written as $p = \rho g h$ (p: hydrostatic pressure, ρ: fluid density, g: gravitational acceleration, h: height of the liquid column). Notably, HP is an isotropic force acting uniformly with equal magnitude in all directions. Therefore, a force that is applied to a fluid in a tube is transmitted through the entire liquid volume to the other end of the tube.

Given the entire human body is based on the interplay of different fluids, almost all cells experience hydrostatic pressure to some extent. However, pressure varies significantly depending on tissue and location in the human body. The interstitial

fluid (0.27 kPa) and cerebrospinal fluid (1.2 kPa) exert pressure at the lower end of the physiologic range (Boulpaep et al. 2009; Irani 2009). Blood pressure which is mainly experienced by endothelial cells lies between 8 and 24 kPa (Klabunde 2011). Osteocytes in the lacunar–canalicular pores of load-bearing cortical bone experience a HP of ~ 270 kPa (Zhang et al. 1998). The upper end of the physiologic range is, for example, found at the femoral head during exercise (~18 MPa) and is more than five orders of magnitude higher than in the interstitial fluid (Morrell et al. 2005). Furthermore, due to compressive loading and unloading during movement, HP increases and decreases frequently with varying magnitude. For example, the human walking frequency is approximately one stride per second (1 Hz), whereas this can easily increase up to three strides per second (>3 Hz) or more during running. Therefore, the physiologic pressure of a specific cell type depends substantially on the location in the body.

The effect of hydrostatic pressure on the differentiation of stem cells is an area of investigation, though more studies deal with the effect of chondrogenic rather than osteogenic differentiation. Bone marrow stem cells are subject to medullary pressure during bone loading. Given bone marrow stem cells give rise to osteoblasts, key players in the bone remodeling process, it seems reasonable that HP plays also an important role in the differentiation process.

Hydrostatic pressure has been found to increase osteogenic and chondrogenic differentiation in terms of mineralization and gene expression although some studies did not observe any effect on stem cell differentiation (Elder and Athanasiou 2009; Steward and Kelly 2015). However, variations in data obtained may occur as a consequence of different experimental setups and loading regimes. Since HP is not constant in the human body, most studies apply not constant but intermittent or cyclic pressure of different frequency and magnitude. Depending on the research question, some studies applied a pressure regime over the entire cultivation period, while other studies chose to apply pressure only once, i.e., at the beginning or end of the experiment. Therefore, it remains unclear what pressure conditions result in either osteogenic or chondrogenic differentiation of adult stem cells. Nevertheless, HP up to 5 MPa was found to increase chondrogenic gene expression, whereas high pressure of 20–50 MPa causes cell apoptosis, tissue disruption, and often a decrease in chondrogenic gene expression.

In general, three parameters can be altered when hydrostatic pressure is applied in cell culture. Firstly, the operation mode: this can be either constant, intermittent (pauses alternating with periods of a specific pressure regime; not necessarily uniform), or cyclic (constant uniform patter). It is also possible to apply a cyclic pattern in an intermittent pressure regime. Secondly, the frequency: this is the rate of increased pressure per second. Usually this parameter is given in the unit Hz, though other units may be used if longer intervals are applied. Thirdly, the magnitude: it defines the maximum pressure and thus the height of amplitude. If pressure is increased and decreased steadily it constitutes sinusoidal wave. Theoretically, it is also possible to alter the rate of increase/decrease and thus the shape of the wave itself.

Various devices have been developed to apply hydrostatic pressure to cell-matrix constructs. Generally, there are two methods to apply HP to a construct. Either the

gas phase of a cell culture vessel is compressed which exerts pressure on the liquid surface (the medium surface) and thus on the construct or, the fluid system itself is compressed with a hydraulic press. If the gas phase is pressurized, the dissolved gas concentration will increase as well. Given most medium buffer systems are based on atmospheric CO_2 regulation of the incubator, pressurization of the gas phase can cause problems in pH control. Especially, if oxygen concentration needs to be kept constant, the gas phase needs to be adjusted. Of course this method might be advantageous when different DO concentrations are required. However, in the second approach only the fluid phase is compressed which allows constant gas concentrations. Usually, the cell culture vessel is connected by tubing to a piston which again is driven by a software controlled hydraulic press. Both approaches require complex setups and often a substantial amount of space, especially when additional pumps for medium exchange or perfusion are necessary.

Most of the bioreactors that are used in current research are custom made and not commercially available. This can be advantageous since innovative bioreactors are developed and optimized by researchers, although results obtained with different bioreactor setups are often not comparable. However, a few commercially available bioreactors exist. The TC-3 by Ebers Medical (Zaragoza, Spain) is a bioreactor with the capability to apply not only HP but also tension, direct compression, and perfusion by modular exchangeable cultivation chambers (Fig. 6). Furthermore, different grips can be used to hold a variety of scaffolds in the bioreactor chamber.

The CartiGen by BISS (Bangalore, India) is also conceptually designed to apply not only HP but also direct compression. Again, cultivation chambers of different sizes to host multiple scaffolds at once are offered. In contrast to the Ebers Medical bioreactor, multiple scaffolds are cultivated in the same chamber, though it is not possible to cultivate multiple samples with independent medium circuits at once.

Although the TC-3 and the CartiGen offer the application of hydrostatic pressure, no publications exist where hydrostatic pressure was applied to stem cells until now.

3.4 Fluid Shear Stress

When a fluid flows over a solid surface, fluid shear forces are exerted on this surface. In a Newtonian fluid like water, the shear stress is proportional to the shear rate which describes the gradient of flow velocity that is perpendicular to the flow direction. Consequently, fluid shear stress describes the deformation of a solid body where a force acts antiparallel to its surface. It is expressed as force per area and has the SI unit $\mathrm{Pa} = \mathrm{N\,m^{-2}} = \mathrm{kg\,m^{-1}\,s^{-2}}$. Another common unit to express fluid shear stress is dyne $\mathrm{cm^{-2}}$ where 1 dyne $\mathrm{cm^{-2}}$ equals 10^{-5} N (kg m $\mathrm{s^{-2}}$) or 0.1 Pa. Since it is a rather small pressure unit (1 Pa equals 1e-5 bar), it is used to express low pressure conditions.

Fluid shear stress is ubiquitous in every living body. About 20% of the body's mass consists of interstitial fluids which is constantly in motion with a speed of approximately 0.1–2 μm $\mathrm{s^{-1}}$ mostly due to osmotic and hydrostatic pressure

differences (Swartz and Fleury 2007). In other tissues like cartilage or bone, it is driven by compressive forces that, for example, are exerted by walking or exercising.

Fluid shear forces are either calculated by computational simulation (computational fluid dynamics (CFD)) or analytical models. Generally, the wall shear stress τ_ω is given by:

$$\tau_\omega = \mu \left(\frac{\partial u}{\partial y} \right)_{y=0} \tag{3}$$

where μ is the viscosity of the fluid, u the flow velocity, y is the distance to the wall, and thus $\delta u/\delta y$ defines the velocity gradient. For simple geometries and boundary conditions, this equation is easily solved. For example, the Hagen-Poiseuille equation describes the shear stress for blood flow in a vessel assuming it is inelastic, cylindrical, and straight and the blood flow is laminar and steady (d = vessel diameter):

$$\tau = 8\mu \frac{u}{d} \tag{4}$$

Yet, calculations become more complex when transferred into a 3D irregular shaped environment. Therefore, CFD simulations that take the actual geometry of the matrix into account are of increasing interest and importance. CFD simulations have solved the Navier-Stokes equation that describes the flow of viscous fluids. For a CFD simulation, a three dimensional model of the matrix is needed as well as the exact starting and boundary conditions. Usually those simulations are precise but take time and require powerful hardware. A simpler approach is the estimation of shear forces with analytical models. They are based on Darcy's law which describes the flow through a porous medium (i.e., a matrix) as a proportional relationship between the flow rate through the medium and the pressure differential over a given distance.

$$Q = \frac{k \cdot A \cdot \Delta P}{\mu \cdot t} \tag{5}$$

In this equation, Q refers to the volumetric flow rate, k is the intrinsic permeability, A is the cross-sectional area, ΔP is the pressure differential of inlet and outlet, μ is the viscosity of the fluid, and t refers to the thickness of the porous medium. From this k can be derived if the other parameters are measured correctly. Darcy's law is only applicable at laminar flow conditions which is true when the corresponding Reynolds number is $Re < 8$. Wang and Tarbell (1995) used an extension of Darcy's law (the Brinkman term) to calculate wall shear stress of smooth muscle cells of the artery wall. If the intrinsic permeability of a scaffold is known, the average shear stress can derived from their model. Other analytical models based on key characteristics of the corresponding scaffold exist, which can be used to approximate shear forces. Analytical models are often optimized on a specific geometry and are not suitable for all scaffolds.

Given shear forces cannot be measured directly, the physiologic range is difficult to estimate and again calculations are often based on models. Therefore, there are limited data detailing physiological in vivo fluid shear stress conditions. However, Weinbaum and Cowin (1994) developed a model in the 1990s for the excitation of osteocytes where the shear stress for osteocytes was estimated to be between 0.8 and 3 Pa. Although exact in vivo shear stress conditions are not known, it is clear that shear forces, even of very low magnitude, have a striking impact on cellular behavior. A number of studies have reported increased cell viability, matrix deposition, and upregulation of differentiation genes when stem cells were exposed to fluid shear stress. As much as the effect of compression and hydrostatic pressure on chondrogenesis is investigated, the effect of fluid shear stress on the osteogenic differentiation of stem cells is subject of current research. Though, in 2D experiments other genes are upregulated than in 3D. Also, in 3D experiment shear forces one order of magnitude lower than predicted by Weinbaum et al. displayed better results and higher shear stress often resulted in cell detachment or apoptosis. Probably, geometric specifications and the arrangement of cells on the respective structure (pores, fibrils, meshes…) have a substantial impact on the actual shear forces that are exerted on the cells which underscores the challenge to calculate actual fluid shear stress.

As it has emerged that fluid shear forces are important in the differentiation of stem cells, many bioreactors have been developed to implement shear forces. Mainly, two different modes of operation can be distinguished. In the first case, the cell-matrix constructs are over flown and not directly perfused. For this, the constructs are placed in a flow chamber where the medium is not forced to pass through the scaffold itself but rather to flow over the scaffold surface. In this case, cells often orientate themselves to the direction of the flow developing a thick cell layer (Maul et al. 2011; Delaine-Smith 2012). Furthermore, depending on the thickness of the scaffold the supply with oxygen and nutrients is not ensured which in return can prohibit the growth of cells into the scaffold. This fluid flow is advantageous for the dynamic cultivation of shear sensitive cells and nonporous scaffolds. Thus, a vast number of custom made bioreactors have been developed that aim to directly perfuse scaffolds at a defined flow rate. These systems often consist of a bioreactor chamber, a medium reservoir, tubing to supply fluids to the chamber, and a peristaltic pump to set a specific flow rate. Various bioreactor chamber geometries were developed to host scaffolds of different stiffness, size, and geometry or to implement additional sensors. The actual shear stress is mainly a product of tube diameter, volumetric flow rate, and the specific characteristics of the scaffold. Therefore, shear stress cannot be monitored and controlled directly with a sensor. For the researcher, if the permeability of a scaffold is known, estimations of the fluid shear stress at different flow rates are possible but must be determined for each bioreactor-scaffold setup. Thus, flow or pressure sensors may be used to confirm if the boundary conditions (inlet flow velocity, volumetric flow rate, or inlet/outlet pressure) used for analytical calculations are constant in the real setup.

The U-Cup bioreactor (Cellec Biotek, Basel, Switzerland) was developed in 2003 and is one of the earliest commercially available perfusion bioreactor systems.

This perfusion bioreactor has been used not only for osteogenic differentiation but also for a wide variety of applications such as generation of vasculogenic grafts, cartilage grafts, expansion of stem cells, cultivation of bone marrow explants, and anticancer compound testing on a 3D tumor model. It is composed of a U-shaped pipe (U-Cup) that can host rigid or soft scaffolds. The pipe is filled with medium and connected to a high precision syringe pump which pumps the medium alternately back and forth with a flow velocity ranging from 1 to more than 10,000 μm s^{-1}. Up to ten independent U-Cups can be placed on a stand and connected to the pump. All bioreactor parts that are in contact with cells are available as disposable components. Two injection ports allow the researcher to directly seed cells into the pipe and use the alternating flow for cell seeding. Dynamic cell seeding procedures were reported to significantly increase seeding efficiency and support a homogeneous cell distribution on the scaffold. Although there are a number of advantages, the system lacks any sensors or the possibility to simultaneously run multiple tubes at different flow rates.

One study has reported on bone marrow derived stem cells that were seeded on a porous scaffold and expanded for 19 days (8.2 fold doublings). The constructs were found to be highly osteoinductive in nude mice compared to constructs seeded with 2D expanded stem cells (Braccini et al. 2005). In a follow-up study, adipose-derived endothelial and osteoblastic progenitor cells were seeded and expanded in the U-Cup system for 5 days and subsequently implanted in nude mice. After 8 weeks an increase in bone and blood vessel formation in the construct compared to constructs with 2D expanded cells was observed (Scherberich et al. 2007). Thus, the U-Cup can be considered as a stable and functional perfusion system for a variety of applications.

3.5 Microfluidic Systems

In the context of cell cultivation under shear stress conditions, microfluidic devices constitute a special class of bioreactor systems. By definition, microfluidics are miniaturized devices that comprise small perfused channels with at least one dimension (typically the channel height or width) in the micrometer range. Due to the small channel geometry, flow regimes within a microfluidic system are always laminar, unless special features such as actuators or surface modifications are implemented (Lee et al. 2011; You et al. 2015). The laminar flow regime does not only ensure continuous supply of nutrients (or any dissolved substances) and removal of waste products but also exerts well-defined mechanical stress on the cells cultivated inside the channels. As stem cells are highly susceptible to shear stress (Adamo and García-Cardeña 2011), microfluidics present a useful technology for research on stem cell differentiation as a consequence of mechanical stimulation (Ertl et al. 2014). The magnitude of the wall shear stress is mainly determined by the channel geometry and flow rate, which allows easy control and regulation. Equation 4 can be used to calculate the wall shear stress in cylindrical channels. However, since the majority of

microfluidic cell culture devices comprises channels with rectangular cross-section, an adapted version is commonly used:

$$\tau_{\omega} = 6\mu \frac{Q}{h^2 w} \tag{6}$$

This equation applies to geometries where the channel width (w) is much bigger than the channel height (h).

Given their small geometry, microfluidic systems are inherently inadequate for production of cells or tissue engineered products, where high cell numbers or large constructs are required. However, the miniaturized format offers several advantages such as minimal consumption of reagents and rapid reaction times. Compared to large bioreactors, higher parallelization and throughput can be achieved (Vyawahare et al. 2010). Furthermore, microfluidic technology is amenable to sensor integration and automation. These features make microfluidics an ideal technology to create testing systems for basic research as well as for drug screening and toxicity testing (Ertl et al. 2014). This is an area of intense research, for example, questions regarding stem cell responses to shear stress, which cannot be investigated in standard cell culture dishes, are often addressed in microfluidic systems. In 2011 Toh et al. presented a microfluidic array in which mouse embryonic stem cells were exposed simultaneously to varying shear stresses of 0.016–16 dyn cm^{-2} (Toh and Voldman 2011). Similarly, effects of low shear stress on stem cell proliferation and osteogenic differentiation were investigated in a microfluidic device where the interstitial fluid flow could be mimicked (Yu et al. 2014). Since stem cells are generally sensitive to mechanical stimulation (resulting in unwanted cell differentiation), special microfluidic systems have been developed to maintain stem cell cultures under low shear stress conditions including physical barriers to separate the supply channels (high shear stress) from the cell compartments (low shear stress) (Lee et al. 2006). Other approaches include the use of laminar flow profile and diffusive mixing to create stable gradients of a test substance while keeping shear forces low (Xu and Heilshorn 2013).

4 Conclusion

This chapter has examined the application of dynamic conditions for the expansion and differentiation of stem cells and the required state-of-the-art bioreactor technology (Table 2). A number of studies have shown the beneficial effects of dynamic conditions on the cultivation of stem cells. Dynamic conditions improve mass transfer in general but are also crucial for the induction of mechanotransductive effects which is favorable for expansion and differentiation of stem cells. In order to develop bioreactor systems that generate in vivo like physiologic conditions, it is evident that the physiologic range of the relevant parameters needs to be identified in advance. Given the expansion of stem cells is a process of low complexity compared to guided differentiation, more bioreactors are commercially available in this field.

Table 2 Characteristics of commercially available bioreactors for the dynamic cultivation of stem cells

Operation mode	Application	Bioreactor system	Scale	Key features	Reference
Orbital shaking	Expansion	Pall Micro-24 MicroReactor System	3–7 ml	24-well cassettes with individual heating, gas injection, pH, and oxygen sensors	www.pall.com
Wave-mixed	Expansion	WAVE Bioreactor family (GE Healthcare Life Sciences)	0.3–500 l	Disposable bags, versions for research, and GMP requirements	(Åkerström 2009; Timmins et al. 2012)
Stirred tank	Expansion	Mobius® product family	3–2000 l	Scale-up and automation	www.emdmillipore.com
Stirred tank	Expansion	Spinner-flasks	125 ml – 36 l	Inexpensive, simple design	(Kumar et al. 2004)
Rotating bed	Expansion, osteogenic differentiation	Z®RP cell cultivation system (Zellwerk GmBH)	2000–8000 cm^2 or up to 12 scaffolds	High degree of automation, GMP compliant	(Diederichs et al. 2009; Neumann et al. 2014)
Perfused	Expansion, co-culture, differentiation	Quantum® Cell Expansion System	2.1 m^2 cell culture surface, large scale	High degree of automation, GMP compliant	www.terumobct.com
Compression	Chondrogenic	CartiGen by BISS	Single-sample (d = 30 mm) – Multi-sample (d = 1.0 mm)	Perfusion can be applied, can be autoclaved	www.tissuegrowth.com

(*continued*)

Table 2 (continued)

Operation mode	Application	Bioreactor system	Scale	Key features	Reference
Tension	Osteogenic differentiation	FX-5000™ Tension	24 well and 6 well formats	Flexible membrane, monolayer cell cultivation	www.flexcellint.com
Tension	Osteogenic differentiation	TC-3 (Ebers Medical)	Up to 3 independent samples,	Ability to stretch scaffolds	www.ebersmedical.com
Hydrostatic pressure	Chondrogenic differentiation	TC-3 (Ebers Medical)	Up to 3 independent samples, pressure up to 0.4 MPa	Compatible with a variety of scaffold shapes and sizes	www.ebersmedical.com
Perfusion/ shear stress	Expansion, differentiation, compound testing	U-Cup bioreactor (Cellec Biotek)	Up to 10 independent samples (Ø 6–10 mm)		(Braccini et al. 2005; Scherberich et al. 2007)
Perfusion/ shear stress	In vitro test models and screening	Microfluidic systems	Microliter scale	High flexibility in design and function, parallelization	(Wu et al. 2011; Ertl et al. 2014)

The advancement of expansion bioreactors is a promising development and a number of bioreactors are already operated in clinical or industrial settings. In contrast, the guided differentiation of stem cells remains a complex, temporal process requiring consideration of a number of parameters. Dependent on the desired tissue, various types of mechanical stimulation are available as well as a diverse range of biomaterials of different size, shape, and surface characteristics which resulting in numerous designs of bioreactors. Furthermore, monitoring of 3D dynamic differentiation processes is more challenging than monitoring of expansion processes. In order to gain a clear and comprehensive knowledge on bioreactor cultivation for the differentiation of stem cells, further research will need to focus on the evaluation of physiologic conditions in the human body, the translation into modular bioreactor technology, and further the systematic screening of cultivation conditions. This would also foster the translation of scientific results to clinical applications to reach the desired goals of "from bench to bedside."

References

Adamo L, García-Cardeña G (2011) Directed stem cell differentiation by fluid mechanical forces. Antioxid Redox Signal 15(5):1463–1473

Åkerström H (2009) Expansion of adherent cells for cell therapy, Uppsala University and GE Healthcare Department of Cell Technologies. Uppsala University, Uppsala

Alimperti S, Lei P et al (2014) Serum-free spheroid suspension culture maintains mesenchymal stem cell proliferation and differentiation potential. Biotechnol Prog 30:974–983

Amit M, Laevsky I et al (2011) Dynamic suspension culture for scalable expansion of undifferentiated human pluripotent stem cells. Nat Protoc 6(5):572–579

Arnsdorf EJ, Tummala P et al (2009) Mechanically induced osteogenic differentiation–the role of RhoA, ROCKII and cytoskeletal dynamics. J Cell Sci 122(4):546–553

Bilgen B, Sucosky P et al (2006) Flow characterization of a wavy-walled bioreactor for cartilage tissue engineering. Biotechnol Bioeng 95:1009–1022

Boulpaep EL, Boron WF et al (2009) Medical physiology a cellular and molecular approach. Signal Transduct 48:27

Braccini A, Wendt D et al (2005) Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts. Stem Cells 23(8):1066–1072

Carrier RL, Rupnick M et al (2002) Perfusion improves tissue architecture of engineered cardiac muscle. Tissue Eng 8:175–188

Chhaya MP, Melchels FPW et al (2015) Sustained regeneration of high-volume adipose tissue for breast reconstruction using computer aided design and biomanufacturing. Biomaterials 52:551–560

Chu L, Robinson DK (2001) Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol 12:180–187

Cierpka K, Elseberg CL et al (2013) HMSC production in disposable bioreactors with regards to GMP and PAT. Chem Ing Tech 85:67–75

Croughan MS, Hamel JF et al (1987) Hydrodynamic effects on animal cells grown in microcarrier cultures. Biotechnol Bioeng 29(1):130–141

Delaine-Smith RM (2012) Mesenchymal stem cell responses to mechanical stimuli. Muscles Ligaments Tendons J 2(3):169–180

Diederichs S, Roker S et al (2009) Dynamic cultivation of human mesenchymal stem cells in a rotating bed bioreactor system based on the Z (R) RP platform. Biotechnol Prog 25(6):1762–1771

Dolley-Sonneville PJ, Romeo LE et al (2013) Synthetic surface for expansion of human mesenchymal stem cells in xeno-free, chemically defined culture conditions. PLoS One 8(8):e70263
Dove A (2013) LIFE SCIENCE TECHNOLOGIES living large: scaling up cell culture. Science 342(6163):1259–1261
Doyle AM, Nerem RM et al (2009) Human mesenchymal stem cells form multicellular structures in response to applied cyclic strain. Ann Biomed Eng 37:783–793
Duncan R, Turner C (1995) Mechanotransduction and the functional response of bone to mechanical strain. Calcif Tissue Int 57(5):344–358
Eghbali H, Nava MM et al (2016) Hollow fiber bioreactor configurations hollow fiber bioreactor technology for tissue engineering applications. Int J Artif Organs 39:1–15
Eibl R, Kaiser S et al (2010) Disposable bioreactors: the current state-of-the-art and recommended applications in biotechnology. Appl Microbiol Biotechnol 86:41–49
Elder BD, Athanasiou KA (2009) Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration. Tissue Eng B Rev 15(1):43–53
Engler AJ, Sen S et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126(4):677–689
Ertl P, Sticker D et al (2014) Lab-on-a-chip technologies for stem cell analysis. Trends Biotechnol 32(5):245–253
Gaut C, Sugaya K (2015) Critical review on the physical and mechanical factors involved in tissue engineering of cartilage. Regen Med 10:1–15
Gey GO (1933) An improved technic for massive tissue culture. Am J Cancer 17(3):752–756
Glacken MW, Fleischaker RJ et al (1983) Large-scale production of mammalian cells and their products: engineering principles and barriers to scale-up. Ann N Y Acad Sci 413:355–372
Goodwin T, Prewett T et al (1993) Reduced shear stress: a major component in the ability of mammalian tissues to form three-dimensional assemblies in simulated microgravity. J Cell Biochem 51(3):301–311
Hao J, Zhang Y et al (2015) Mechanobiology of mesenchymal stem cells: perspective into mechanical induction of MSC fate. Acta Biomater 20:1–9
Hervy M, Weber JL et al (2014) Long term expansion of bone marrow-derived hMSCs on novel synthetic microcarriers in xeno-free, defined conditions. PLoS One 9(3):e92120
Hodge W, Fijan R et al (1986) Contact pressures in the human hip joint measured in vivo. Proc Natl Acad Sci 83(9):2879–2883
Hyun I (2010) The bioethics of stem cell research and therapy. J Clin Invest 120(1):71–75
Irani DN (2009) Cerebrospinal fluid in clinical practice. Elsevier Health Sciences, Philadelphia
Jayapal KP, Wlaschin KF et al (2007) Recombinant protein therapeutics from CHO cells-20 years and counting. Chem Eng Prog 103(10):40
Jeon JE, Schrobback K et al (2012) Dynamic compression improves biosynthesis of human zonal chondrocytes from osteoarthritis patients. Osteoarthr Cartil 20:906–915
Johnson GA, Tramaglini DM et al (1994) Tensile and viscoelastic properties of human patellar tendon. J Orthop Res 12:796–803
Jossen V, Pörtner R, Kaiser SC, Kraume M, Eibl D and Eibl R (2014) Mass Production of Mesenchymal Stem Cells — Impact of Bioreactor Design and Flow Conditions on Proliferation and Differentiation, Cells and Biomaterials in Regenerative Medicine Daniel Eberli, IntechOpen, https://doi.org/10.5772/59385. Available from: https://www.intechopen.com/books/cells-and-biomaterials-in-regenerative-medicine/mass-production-of-mesenchymal-stem-cells-impact-of-bioreactor-design-and-flow-conditions-on-prolife
Kaiser SC, Eibl R et al (2011) Engineering characteristics of a single-use stirred bioreactor at bench-scale: the Mobius CellReady 3L bioreactor as a case study. Eng Life Sci 11:359–368
Kearney EM, Farrell E et al (2010) Tensile strain as a regulator of mesenchymal stem cell osteogenesis. Ann Biomed Eng 38:1767–1779
Kisiday JD, Frisbie DD et al (2009) Dynamic compression stimulates proteoglycan synthesis by mesenchymal stem cells in the absence of chondrogenic cytokines. Tissue Eng A 15(10):2817–2824
Klabunde R (2011) Cardiovascular physiology concepts. Lippincott Williams & Wilkins, Philadelphia

Kumar S, Wittmann C et al (2004) Minibioreactors. Biotechnol Lett 26:1–10
Kunas KT, Papoutsakis ET (1990) Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment. Biotechnol Bioeng 36(5):476–483
Laurent LC, Ulitsky I et al (2011) Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. Cell Stem Cell 8(1):106–118
Lechanteur C (2014) Large-scale clinical expansion of mesenchymal stem cells in the GMP-compliant, closed automated quantum® cell expansion system: comparison with expansion in traditional T-flasks. J Stem Cell Res Ther 04:222
Lee PJ, Hung PJ et al (2006) Nanoliter scale microbioreactor array for quantitative cell biology. Biotechnol Bioeng 94(1):5–14
Lee C-Y, Chang C-L et al (2011) Microfluidic mixing: a review. Int J Mol Sci 12(5):3263–3287
Li J, Wang J et al (2012) The influence of delayed compressive stress on TGF-β1-induced chondrogenic differentiation of rat BMSCs through Smad-dependent and Smad-independent pathways. Biomaterials 33:8395–8405
Lopes AG (2015) Single-use in the biopharmaceutical industry: a review of current technology impact, challenges and limitations. Food Bioprod Process 93:98–114
Luciani N, Du V et al (2016) Successful chondrogenesis within scaffolds, using magnetic stem cell confinement and bioreactor maturation. Acta Biomater 37:101–110
Maul TM, Chew DW et al (2011) Mechanical stimuli differentially control stem cell behavior: morphology, proliferation, and differentiation. Biomech Model Mechanobiol 10(6):939–953
McMahon LA, Reid AJ et al (2008) Regulatory effects of mechanical strain on the chondrogenic differentiation of MSCs in a collagen-GAG scaffold: experimental and computational analysis. Ann Biomed Eng 36(2):185–194
Mohamet L, Lea ML et al (2010) Abrogation of E-cadherin-mediated cellular aggregation allows proliferation of pluripotent mouse embryonic stem cells in shake flask bioreactors. PLoS One 5(9):e12921
Moretti M, Prina-Mello A et al (2004) Endothelial cell alignment on cyclically-stretched silicone surfaces. J Mater Sci Mater Med 15:1159–1164
Morrell KC, Hodge WA et al (2005) Corroboration of in vivo cartilage pressures with implications for synovial joint tribology and osteoarthritis causation. Proc Natl Acad Sci U S A 102(41):14819–14824
Neumann A, Lavrentieva A et al (2014) Characterization and application of a disposable rotating bed bioreactor for mesenchymal stem cell expansion. Bioengineering 1(4):231–245
Nold P, Brendel C et al (2013) Good manufacturing practice-compliant animal-free expansion of human bone marrow derived mesenchymal stroma cells in a closed hollow-fiber-based bioreactor. Biochem Biophys Res Commun 430:325–330
Noyes FR, Grood ES (1976) The strength of the anterior cruciate ligament in humans and Rhesus monkeys. J Bone Joint Surg 58:1074–1082. American volume
Oh SK, Chen AK et al (2009) Long-term microcarrier suspension cultures of human embryonic stem cells. Stem Cell Res 2(3):219–230
Pelaez D, Arita N et al (2012) Extracellular signal-regulated kinase (ERK) dictates osteogenic and/or chondrogenic lineage commitment of mesenchymal stem cells under dynamic compression. Biochem Biophys Res Commun 417:1286–1291
Reinders ME, de Fijter JW et al (2013) Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. Stem Cells Transl Med 2(2):107–111
Reinders ME, Bank JR et al (2014) Autologous bone marrow derived mesenchymal stromal cell therapy in combination with everolimus to preserve renal structure and function in renal transplant recipients. J Transl Med 12(1):1
Reuveny S, Bino T et al (1979) Pilot plant scale production of human lymphoblastoid interferon. Dev Biol Stand 46:281–288
Rojewski MT, Fekete N et al (2013) GMP-compliant isolation and expansion of bone marrow-derived MSCs in the closed, automated device quantum cell expansion system. Cell Transplant 22:1981–2000

Rui YF, Lui PPY et al (2011) Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. J Orthop Res 29(3):390–396
Schechtman H, Bader DL (1997) In vitro fatigue of human tendons. J Biomech 30:829–835
Scherberich A, Galli R et al (2007) Three-dimensional perfusion culture of human adipose tissue-derived endothelial and osteoblastic progenitors generates osteogenic constructs with intrinsic vascularization capacity. Stem Cells 25(7):1823–1829
Schnitzler AC, Verma A et al (2016) Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: current technologies and challenges. Biochem Eng J 108:3–13
Singh V (1999) Disposable bioreactor for cell culture using wave-induced agitation. Cytotechnology 30(1–3):149–158
Somerville RP, Devillier L et al (2012) Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE® bioreactor. J Transl Med 10(1):1
Steward AJ, Kelly DJ (2015) Mechanical regulation of mesenchymal stem cell differentiation. J Anat 227(6):717–731
Storm MP, Sorrell I et al (2016) Hollow fiber bioreactors for in vivo-like mammalian tissue culture. J Vis Exp 111:1–12. https://doi.org/10.3791/53431
Subramony SD, Dargis BR et al (2013) The guidance of stem cell differentiation by substrate alignment and mechanical stimulation. Biomaterials 34(8):1942–1953
Sun L, Qu L et al (2016) Effects of mechanical stretch on cell proliferation and matrix formation of mesenchymal stem cell and anterior cruciate ligament fibroblast. Stem Cells Int 2016:1–10
Swartz MA, Fleury ME (2007) Interstitial flow and its effects in soft tissues. Annu Rev Biomed Eng 9:229–256
Timmins NE, Kiel M et al (2012) Closed system isolation and scalable expansion of human placental mesenchymal stem cells. Biotechnol Bioeng 109(7):1817–1826
Toh Y-C, Voldman J (2011) Fluid shear stress primes mouse embryonic stem cells for differentiation in a self-renewing environment via heparan sulfate proteoglycans transduction. FASEB J 25(4):1208–1217
Tran SC, Cooley AJ et al (2011) Effect of a mechanical stimulation bioreactor on tissue engineered, scaffold-free cartilage. Biotechnol Bioeng 108:1421–1429
Trounson A, McDonald C (2015) Stem cell therapies in clinical trials: progress and challenges. Cell Stem Cell 17(1):11–22
Tyler JE (1990) Microencapsulation of mammalian cells. Bioprocess Technol 10:343–361
Usuludin SBM, Cao X et al (2012) Co-culture of stromal and erythroleukemia cells in a perfused hollow fiber bioreactor system as an in vitro bone marrow model for myeloid leukemia. Biotechnol Bioeng 109:1248–1258
Van Wezel A (1967) Growth of cell-strains and primary cells on micro-carriers in homogeneous culture. Nature 216:64–65
Vick Roy TB, Blanch HW et al (1983) Microbial hollow fiber bioreactors. Trends Biotechnol 1:135–139
Vournakis J, Runstadler P Jr (1990) Optimization of the microenvironment for mammalian cell culture in flexible collagen microspheres in a fluidized-bed bioreactor. Biotechnology (Reading, Mass) 17:305–326
Vyawahare S, Griffiths AD et al (2010) Miniaturization and parallelization of biological and chemical assays in microfluidic devices. Chem Biol 17(10):1052–1065
Wang D, Tarbell J (1995) Modeling interstitial flow in an artery wall allows estimation of wall shear stress on smooth muscle cells. J Biomech Eng 117(3):358–363
Weinbaum S, Cowin S et al (1994) A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech 27(3):339–360
Wu H-W, Lin C-C et al (2011) Stem cells in microfluidics. Biomicrofluidics 5(1):013401
Wung N, Acott SM et al (2014) Hollow fibre membrane bioreactors for tissue engineering applications. Biotechnol Lett 36:2357–2366
Xu H, Heilshorn SC (2013) Microfluidic investigation of BDNF-enhanced neural stem cell chemotaxis in CXCL12 gradients. Small 9(4):585–595

Yassin MA, Leknes KN et al (2016) Surfactant tuning of hydrophilicity of porous degradable copolymer scaffolds promotes cellular proliferation and enhances bone formation. J Biomed Mater Res A 104:2049–2059

You JB, Kang K et al (2015) PDMS-based turbulent microfluidic mixer. Lab Chip 15(7):1727–1735

Yu W, Qu H et al (2014) A microfluidic-based multi-shear device for investigating the effects of low fluid-induced stresses on osteoblasts. PLoS One 9(2):e89966

Zeilinger K, Schreiter T et al (2011) Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies. Tissue Eng Part C Methods 17:549–556

Zhang D, Weinbaum S et al (1998) Estimates of the peak pressures in bone pore water. J Biomech Eng 120(6):697–703

Zhang Z-Y, Teoh SH et al (2009) A biaxial rotating bioreactor for the culture of fetal mesenchymal stem cells for bone tissue engineering. Biomaterials 30(14):2694–2704

Zhang Z-Y, Teoh SH et al (2010) A comparison of bioreactors for culture of fetal mesenchymal stem cells for bone tissue engineering. Biomaterials 31(33):8684–8695

Zhao W, Li X et al (2014) Effects of substrate stiffness on adipogenic and osteogenic differentiation of human mesenchymal stem cells. Mater Sci Eng C 40:316–323

Hard Material Modulation for (Skeletal) Tissue Engineering Purposes

Paul E. Bourgine

Contents

Abstract

Scaffolding materials are three-dimensional (3D) structures derived from existing tissue (e.g., decellularized bone, collagen-based) or de novo synthesized (e.g., titanium, polycaprolactone), supporting cell attachment, proliferation, and differentiation. Despite the increasing number of biocompatible templates, few of them represent a gold standard treatment for regenerative medicine. This drives the development of advanced materials with increased performance. Nowadays, the diversification of isolation and/or fabrication methods offers a wide potential to design specific structures with tailored properties. In this chapter, we present the main existing strategies to customize 3D material properties toward optimized tissue regeneration. The possibility to tune the composition/architecture as well as to functionalize materials with bioactive elements will be discussed.

P. E. Bourgine (✉)
Cell, Tissue and Organ Engineering Laboratory, Department of Clinical Sciences Lund, Lund University, Lund, Sweden

Lund Stem Cell Centre, Lund University, Lund, Sweden

Wallenberg Centre for Molecular Medicine, Lund University, Lund, Sweden
e-mail: paul.bourgine@med.lu.se

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_30

1 Introduction

By definition, tissue engineering combines the use of stem and/or committed cells with scaffolding material supporting cell growth/differentiation, toward the generation of a graft. Nowadays, a large variety of materials are currently available for regenerative medicine purposes (Table 1). This includes synthetic ceramics, polymers, and metals but also biologically derived substrates (Badylak 2007). While their utility for replacement of mechanical function is a proven success in a certain number of scenarios (e.g., teeth, hips, knees, heart valves, intervertebral discs) (Williams 2009; Chen and Thouas 2015; Hench 1991), the regeneration of host tissues driven by materials remains challenging. Indeed, considering the diverse roles carried on by a tissue, it makes unlikely that the regeneration and subsequent restoration of the ensued functions can be achieved by the use of a simple material (Hutmacher et al. 2007). Nevertheless, one can identify and consider key characteristics such as the composition, architecture, structural mechanics, surface properties, or biological properties (e.g., biocompatibility/biodegradability), in order to envision and develop suitable material for a specific regeneration context. In fact, this drives the development of advanced materials capable to increase tissue regeneration by customization of their properties.

In vivo, cells interact with their surrounding environment and permanently receive cues which determine their fate (Berrier and Yamada 2007; Frantz et al. 2010). In particular, the extracellular matrix (ECM) plays a key role as primary cellular substrate acting as structural niche, growth factor storage, and presenting

Table 1 Typical examples of scaffolds derived from existing tissues (biologic) or de novo synthetized (synthetic) used in regenerative medicine (Ehrbar et al. 2008; LeGeros 2008; Dhandayuthapani et al. 2011)

Scaffold	Composition	Properties	Origin
Ceramic	CaP, hydroxyapatite	Bioactive, biocompatible, biodegradable,	Biologic, synthetic
Extracellular matrix	Collagen type 1, glycosaminoglycans	Bioactive, biodegradable,	Biologic
Metals	Titanium, nickel, steel	Bioinert, biocompatible	Synthetic
Polymeric	Polycaprolactone, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers PLGA	Biocompatible	Synthetic
Bioactive glass	CaO-Na_2O-SiO_2, P_2O_5	Bioactive, biodegradable, Biocompatible	Synthetic
Hydrogels	Fibrin, cellulose, chitosan, polyester, silicon	Bioactive, biocompatible, biodegradable	Biologic, synthetic
Composite materials	Mixed	Biocompatible	Synthetic

interface (Frantz et al. 2010). The composition of the ECM and its mechanical strength are intertwined parameters that largely influence cellular behaviors, driving tissue formation, regeneration, and homeostasis.

Similarly, cell-material interactions have been shown to directly regulate adhesion, proliferation, and differentiation of the cells (Engler et al. 2006; Guilak et al. 2009). In the context of tissue engineering, this directly influences the cell seeding efficiency, cell colonization/distribution, and graft maturation, respectively. As such, the modulation of scaffold's composition, architecture, but also its functionalization with bioactive elements offers the attractive possibility to impact cell behavior accordingly, toward generation of grafts with increased properties and ultimately enhanced efficacy of tissue repair.

In this chapter, we focus on the main existing strategies to modulate 3D hard scaffolding material (thus excluding gel-like structures) properties for tissue engineering purposes. First, we will present the possibility to modulate the primary properties of a material by modification of composition/mechanical strength/architecture. We will then discuss the existing opportunities to further tune structures by functionalization with bioactive elements. Content will be largely exemplified by specific studies derived from the skeletal tissue engineering context.

2 Primary Material Properties Modulation

The choice of a scaffolding material is typically done in consideration of the targeted tissue, in order to possibly approach its biological and physical properties (Fig. 1). A large range of materials are subject to composition and architectural modifications in order to optimize and adjust these parameters. This includes stiffness, biodegradability, or simply geometrical changes.

2.1 Biological and Physical Modulation

The mechanical properties of 3D materials can be tuned by direct modification of their composition. This is generally achieved by the molecular modulation of composition of synthetic scaffolds or by generation of composite structures in order to generate a novel material with adequate properties. Such hybrid composition appears to be a valid option regarding scaffolding materials derived from biological tissues (e.g., decellularized bone) since their primary composition can hardly be changed as opposed to their synthetic counterpart.

Thus, a large number of studies involved the use of hybrid constructs such as collagen-glycosaminoglycans (GAG) (Murphy et al. 2012), collagen-chitosan (Bi et al. 2011), or silk-collagen (Shen et al. 2014) composites. In bone tissue engineering, this can be exemplified by incorporation of calcium phosphate particles increasing the osteoinductivity of the material (Guillaume et al. 2017). Similarly, the combination of poly(lactide-co-glycolide) (PLGA) scaffolds with calcium phosphate elements was used to optimize the formation of tooth tissue from human dental

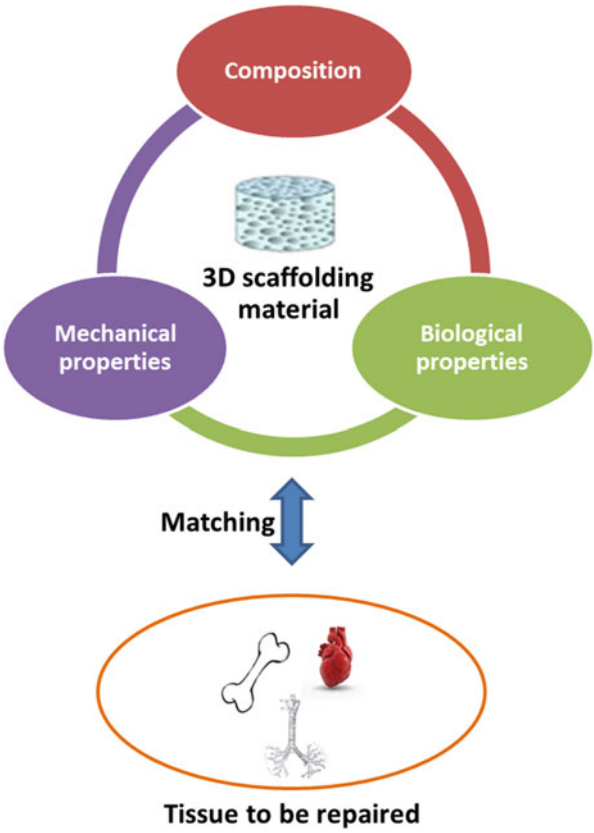


Fig. 1 Correlations between biological and physical parameters of material and tissue. The composition, mechanical, and biological parameters of a material are intertwined parameters, their modulation impacting on each other's property. Typically, while designing a material-based strategy for tissue repair, the graft properties should approach/match those of the target tissue

pulp stem cells (Zheng et al. 2011). The same strategy was used but combining collagen and hydroxyapatite, this directly impacting on the in vivo promotion of bone formation (Rodrigues et al. 2003).

The modulation of composition has a direct impact on the mechanical properties of a material, including its stiffness. Since cells are biological sensors capable to read and react to substrate stiffness (Discher et al. 2005), the modulation of this parameter has been investigated to influence the proliferation but also the differentiation of cells (Engler et al. 2006). On 3D scaffolding materials, this was shown by influencing mesenchymal stromal cells and osteoblast proliferation/differentiation using GAG-collagen structures of various stiffness (Murphy et al. 2012; Haugh et al. 2011). Impact of stiffness modulation can be further exemplified with the gradual addition of hydroxyapatite (Guillaume et al. 2017) to collagen-based scaffolds significantly increasing scaffold stiffness and subsequently associated with an improved in vitro and in vivo osteogenesis (Gleeson et al. 2010).

Closely related to stiffness, the elastic modulus (deformation capacity of a substrate) is an important parameter taken into account toward the generation of a graft. In the skeletal regeneration context, this is exemplified by the use of ceramics

approaching the natural composition and mechanical properties of bone (LeGeros 2008). In this regard, a recent study reported the successful fabrication of glass-ceramic scaffold matching the mechanical strength of cortical bone (Roohani-Esfahani et al. 2016).

Importantly, the modulation of scaffolds composition is also a mean to regulate its degradation rate, which is essential to ensure primary engraftment (vascularization/remodeling) and mechanical stability prior to its progressive replacement by host tissue. The control over degradability impacts the graft remodeling into tissue of ideally equal quality/properties than pre-trauma. Ideally, a controllable degradation and restoration rates should match the rate of tissue growth in vitro and in vivo for biodegradable or restorable materials. However, slow degradable materials can also present some advantages in particular scenarios, in order to provide biological stability for permanent support over time (Dhandayuthapani et al. 2011).

Toward modulation of degradability, polymers are interesting materials as backbones of definable molecular weight easily combinable, thus offering flexibility over their degradation rate (Martina and Hutmacher 2007). This has given tissue engineers the flexibility to tailor the mechanical and degradation properties of a material for specific applications. The biodegradation of polymeric biomaterials involves cleavage of hydrolytically or enzymatically sensitive bonds in the polymer leading to its erosion (Katti et al. 2002). In practice, incorporation of poly(ethylene oxide) in PCL-based scaffolds was shown to influence biodegradability through capacity of water uptake (Huang et al. 2004). Another example consists in the generation of an amino acid ester polyphosphazene/hydroxyapatite composite for bone regeneration. The tuning of degradation was achieved by incorporation of side chains making the polymer backbone more or less sensitive to hydrolysis (Ambrosio et al. 2002).

For biological materials, degradation modulation can be achieved by cross-linking of collagen fibrils. This has been demonstrated to not only influence the stiffness of the resulting materials but also their degradation rate (Depalle et al. 2015; Grover et al. 2012).

2.2 Material Properties Modulation by Geometry Changes

In vivo, the microstructure of the ECM varies from tissue to tissue. For instance, the pore size ranges from tens to hundreds of nanometers in dense connective tissues to hundreds of microns in dermis. Since the ECM is typically not a homogenous substrate, the size, shape, and volume of the pores affect the rate of diffusion of soluble factors to cells. Thus, the given geometry of a scaffold can be critical in defining bioactivity, and influence the distribution and invasion of cells, or subsequently its vascularization. The modulation of scaffolds architecture has been investigated as a strategy to modify biological performance of materials, by tailoring their macro- or micro-architecture.

In this direction, the pore size of 3D collagen-GAG scaffolds was shown to influence the migratory behavior of fibroblasts in vitro, illustrated by the decreased cellular motility when pore size increased (Harley et al. 2008). In

another study, collagen-hyaluronic acid scaffolds of different pore sizes were shown to differentially stimulate in vitro chondrogenesis of MSC (Matsiko et al. 2014), thus having implications for the generation of cartilage substitutes. Similarly, scaffolds of different geometrical structures were shown to influence the cell seeding efficiency and distribution of in vitro grafts. Using scaffolds of different designs, dynamically seeded using perfusion bioreactors, a correlation between regions of high cell densities and presence of larger pores was identified (Melchels et al. 2011).

Scaffolds architecture was also shown to have implications on its in vivo performance. For instance, in an in vivo model of nerve regeneration, collagen tubular structures were investigated for their capacity to reconnect transected peripheral nerve (Sannino and Madaghiele 2009). By micro-patterning (modulation of pore size and orientation) of the material, the regenerative potential of the conduit could be enhanced. This was linked with the increased cellular permeability, guiding the growth of neural structures across the injury site. Altogether, these examples illustrate the importance of micro-architecture as a parameter to be considered in order to modulate and design new materials.

In addition, nowadays the use of 3D printing technologies represents a promising strategy to allow generation of scaffolds with more precise geometry (Ovsianikov et al. 2018; Murphy and Atala 2014; see also Volume *3D Printing and Biofabrication* which is part of the *Tissue Engineering and Regeneration* book series (https://doi.org/10.1007/978-3-319-45444-3)). Printing of calcium phosphate was thus investigated for fabrication of bone grafts with a resolution of 0.5 mm (Inzana et al. 2014). This can obviously be combined with the 3D special patterning of cells and/or molecules, as illustrated by a BMP-2 and VEGF spatial distribution on PCL scaffolds (Park et al. 2015). Together with computer-aided scaffold design (Chua et al. 2005), already used for the design of bone substitutes (Grayson et al. 2010), the damaged tissue could be imaged and subsequently lead to the fabrication of tissue with both their micro- and macrostructure precisely designed (Fig. 2).

More provocatively, the global design of a material could be considered in order to trigger organomorphic properties (Toni et al. 2011). In other words, conceptually this implies that the growth and differentiation of seeded cells are influenced by the 3D scaffold geometry per se, prompting the organization and subsequently fate of seeded cells.

3 Material Functionalization

The functionalization of a material consists in enhancing its basic biological properties by improving/adding a defined function to the pre-existing structure. This can be achieved by combining scaffolds with biological or chemical elements. The resulting functionalized material can either be subsequently seeded with cells, thus serving as new in vitro substrate – toward the generation of a graft – or directly implanted as smart material capable to induce per se endogenous mechanisms of

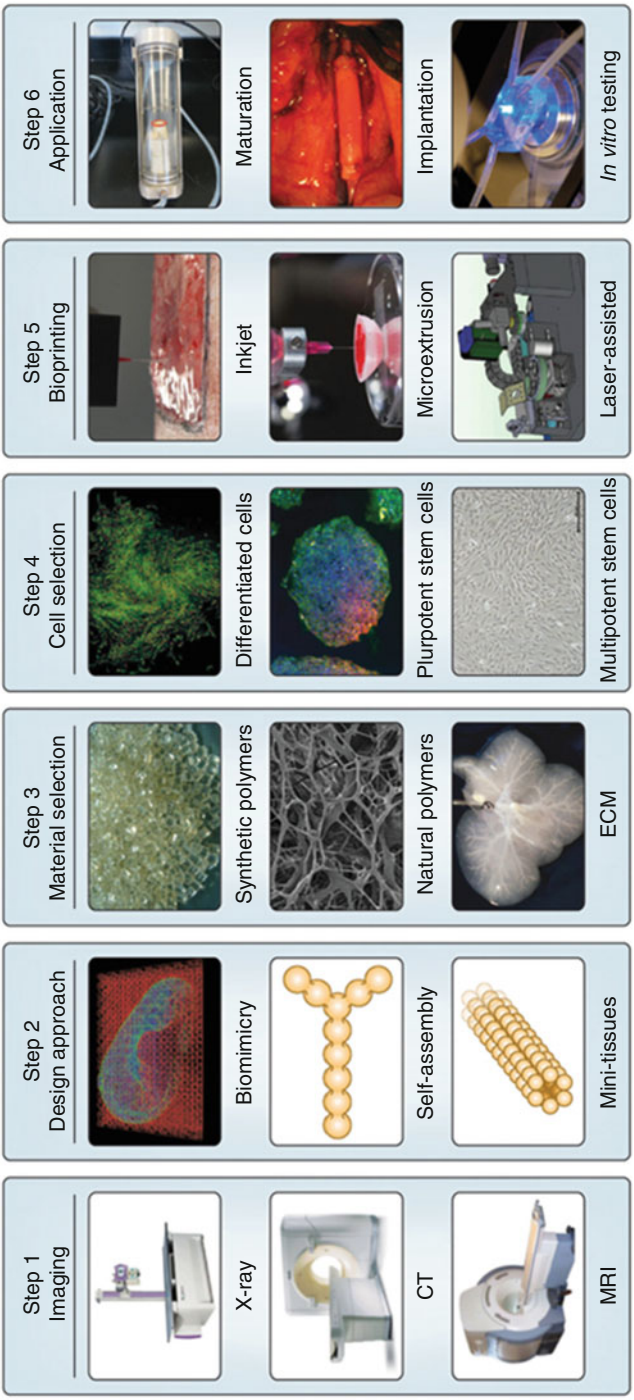


Fig. 2 Strategy for the ultimate generation of a graft. The defect site is imaged (step 1) to help the design of suitable selected materials with particular microarchitecture (step 2 and step 3). The resulting customized 3D material is seeded with allogeneic or autologous cells (step 4) with or without the help of printing technologies (step 5). The resulting constructs can follow a maturation step (typically for the generation of a tissue by seeded cells), tested for its properties in vitro or directly implanted at defect site. (From Murphy and Atala (2014))

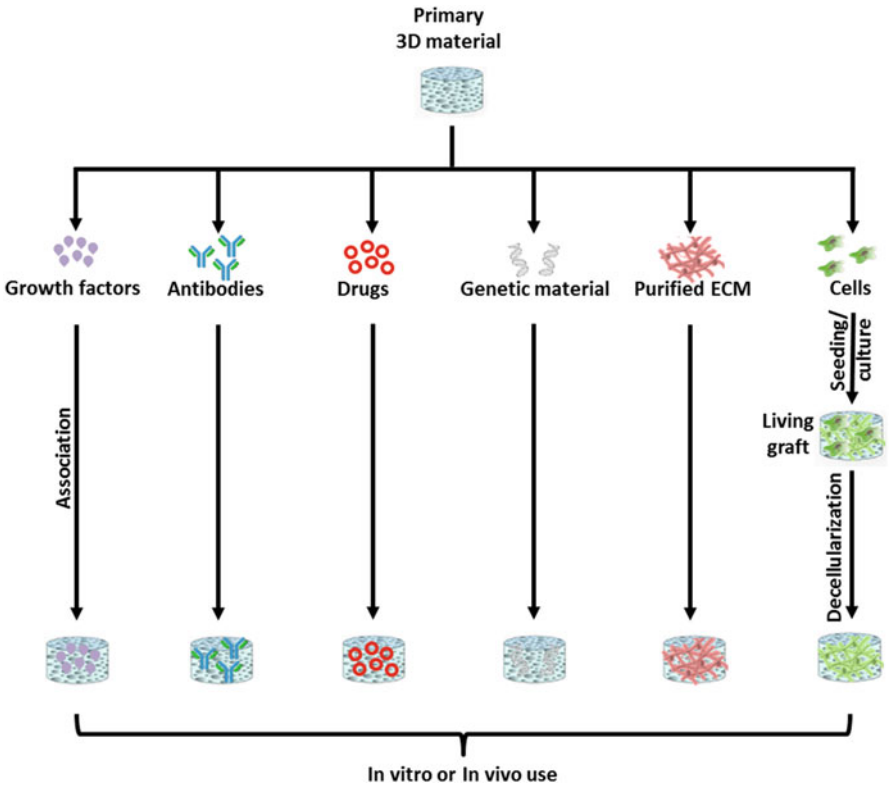


Fig. 3 Functionalization of materials with bioactive elements. Primary materials can be associated with a certain number of biological (e.g., protein, genetic material) or chemical (e.g., pharmaceutical drugs) factors in order to improve their basic properties. The different strategies can be combined (e.g., growth factors immobilized on purified ECM-scaffold hybrid) for advanced customization of the primary material. The resulting constructs can then be used for in vitro or in vivo applications

tissue repair. Here, we distinguish the functionalization resulting from binding of bioactive factors from the ECM-based scaffold ornamentation (Fig. 3).

3.1 Material Functionalization by Association with Bioactive Factors

3.1.1 Growth Factor Functionalization

Bioactive elements can be chemically immobilized or physically encapsulated into specific materials, to be ultimately released by simple diffusion or progressive tissue degradation/remodeling (Lee et al. 2000). Thus, the resulting scaffold conjugated with the factor(s) of interest serves as vehicle for its controlled release. Methods of factor incorporation principally include covalent bonding, chemical immobilization,

physical adsorption, encapsulation, entrapment, or even electrospinning technologies (Yoo et al. 2009). Together with the possibility to tune material property/composition, the strategy offers an appealing control over the factor release profile for optimized concentrations and kinetic of delivery.

Tissue development and regeneration events are driven by growth factors controlling migration, proliferation, and differentiation but also survival of cells (Cross and Dexter 1991). The progressive identification of the main factors contributing to tissue formation events allowed investigating their association with material for targeted tissue repair.

First approaches included the use of the promitotic basic fibroblast growth factor (bFGF) (Edelman et al. 1991), vascular endothelial growth factor (VEGF) (Ferrara et al. 2003a), and platelet-derived growth factor (PDGF) as strong regulator of angiogenesis (Heldin and Westermark 1999) or bone morphogenetic proteins (BMPs) as potent inducer of bone formation (Reddi 1998).

Delivery of VEGF by polymeric scaffolds (Sheridan et al. 2000; Murphy et al. 2000) successfully allowed the sustained release of the growth factor, with preservation of bioactivity leading to promotion of angiogenesis in vivo (Lee et al. 2000). Similarly, association of BMP-2 with collagen sponge (Friess et al. 1999; Hollinger et al. 1998) resulted in delivery and increased bone formation in a rabbit model.

Primary successes in growth factor-scaffold association in terms of delivery and measurable biological effects were nuanced by observed drawbacks. In fact, initial studies relied at first on the use of a single and potent factor, sufficient in triggering the targeted tissue formation process. However, this was achieved by local delivery of substantial doses, rapidly shown to be associated with major complications, such as BMPs and VEGF, respectively, with ectopic site bone formation (Carragee et al. 2011) and development of aberrant vessels (Ozawa et al. 2004). This implies the need for a superior control over the kinetic of release in order to reduce instantaneous doses while extending the delivery in time. This was actually shown to be of primary importance in the context of therapeutic angiogenesis, where the dose of VEGF should be controlled and sustained over a 4 weeks period for establishment of stable vasculature (Sacchi et al. 2014).

Beyond kinetic control, combining scaffolds with multiple growth factors was rapidly adopted with the rationale that single molecules do not promote complex regeneration processes with the same efficiency as a cocktail of factors (Richardson et al. 2001). This "multiplexing approach" may allow reducing the possibly unsafe total doses of delivered growth factors. In this direction, the combined delivery of VEGF and PDGF from polymers was first investigated and successfully led to mature formation of vasculature network. On the same line, poly(lactide-co-glycolide) (PLG)-based scaffolds releasing up to three factors (VEGF, hepatocyte growth factor, and angiopoietin-1) were shown to more efficiently enhance angiogenesis in a murine hind limb ischemia model (Saif et al. 2010).

Combining factors was also considered to induce different types of processes simultaneously for increased beneficial effects. For instance, the combination of VEGF and BMP-2 was performed toward achieving both enhanced construct

engraftment and efficiency of bone formation, in a sheep model (Suárez-González et al. 2014).

In addition to growth factors, other proteins can be effectively combined with materials and lead to its functionalization. This includes antibodies, exemplified by delivery of TNFα antibody associated with chitosan-collagen scaffold to control local inflammation over time (Wang et al. 2015). Small oligopeptides are also commonly used in the form of arginine-glycine-aspartic acid (RGD) domains favoring cell adhesion and matrix metalloproteinase (MMP)-sensitive sequences (Lutolf et al. 2003) but also peptides with particular binding affinity for specific growth factors (Maynard and Hubbell 2005), thus intrinsically regulating their kinetic of release.

3.1.2 Drugs/DNA/RNA Functionalization

Chemical compounds (e.g., serving as pharmaceutical drugs) are used to functionalize material and increase their efficacy of delivery/action. Polycaprolactone (PCL) scaffolds were successfully combined with an antibiotic (Biteral®) by electrospinning, preventing post-surgery bacterial infection and improving healing in rats.

Interestingly, this strategy is not necessarily used in view of promoting tissue healing/formation but also to possibly prevent vascularization/remodeling of an implanted tissue to preserve its integrity. This was illustrated by incorporation of the anti-angiogenic drug Avastin® into in vitro-generated cartilage templates, blocking the otherwise impending remodeling process into bone occurring upon ectopic implantation in mouse (Centola et al. 2013), thus detrimental to stability of cartilage tissue. Obviously, scaffold-based delivery of anticancer drugs is also of primary interest and consequently under investigation for treatment of various tumors using silk and polymeric scaffolds (Anaraki et al. 2015; Zhan et al. 2013; Tian et al. 2014) but goes beyond the main scope of this chapter.

Another approach relies on scaffolds loaded with genetic sequences in the form of DNA or RNA. The scaffold structure is used as transfection vector for a targeted gene delivery to either in vitro seeded cells or to local tissue in vivo. The transfected sequence may encode for a protein that will be continuously expressed and released, stimulating cell differentiation/proliferation or tissue morphogenesis. As opposed to scaffolds functionalized with growth factors, this strategy avoids protein instability issues associated with the severe formulation process and their short half-life observed after release in the body fluid (Chen and Mooney 2003; Chung and Park 2007). Early studies include the delivery of PDGF-encoding plasmid DNA, in association with PLG scaffolds. This was shown to achieve successful and efficient cell transfection in vivo and induced matrix deposition and vascularization (Shea et al. 1999). Another group exemplified the possibility of scaffold functionalization for in vitro purposes, illustrated by combination of transforming growth factor β (TGFβ) with chitosan-gelatin structures, leading to enhanced proliferation of chondrocytes after seeding.

3.2 ECM-Based Material Functionalization

The ECM is the acellular component of our tissues and possesses important structural, mechanical, and functional roles. Its primary composition directly correlates with the elasticity/stiffness of a tissue, while its high binding affinity for growth factor functions as reservoir cues instructing tissue morphogenesis, differentiation, and homeostasis (Frantz et al. 2010). Primary constituents of the ECM consist in collagens, fibronectin, laminin, and elastin, in diverse proportions largely depending on the tissue of origin. Scaffold functionalization by ECM can be distinguished into purification and subsequent scaffold coating with ECM-derived protein or the cellular-driven deposition of ECM followed by a decellularization process.

3.2.1 Materials Coating with Purified ECM

Despite existence of scaffolds already consisting per se in purified and tailored ECM (Badylak 2007), (e.g., collagen type 1), hard materials can be further coated with ECM. Here we focused on the combination of rigid structure functionalized by an additional coating of ECM protein.

Typical coatings consist in "structural" ECM components, providing an ideal surface to promote cell adhesion, migration, and proliferation. (Thus this scaffold functionalization enhances the material basic properties toward the rationale to facilitate the tissue regeneration process (Lutolf 2005).) Ideally the coated ECM also act as substrate to be used by cells for tissue formation (e.g., collagens for bone regeneration). Importantly, despite principally considered and described as proteins with a structural role, collagens but also fibronectin has been recently associated with mechanisms of tissue remodeling, longevity, and regeneration (Lukjanenko et al. 2016; Ewald et al. 2015), thus going beyond the merely architectural role predominantly reported.

Fibronectin coating of porous polymers was rapidly demonstrated to promote chondrocytes proliferation (Bhati et al. 2001). In the context of peripheral nerve regeneration, laminin has been successfully associated with collagen-based scaffolds which ultimately resulted in improved nerve regeneration in rats (Cao et al. 2011). Collagen, being the most abundant ECM protein (Frantz et al. 2010), has also been massively employed for scaffolding functionalization (Douglas and Haugen 2008). This is particularly the case toward bone regeneration strategy, illustrated by the enhancement of osteogenic capacity of titanium scaffolds in vitro (Roehlecke et al. 2001) or by the increased bone mass repair induced in rat model (Wojtowicz et al. 2010).

The affinity of typically coated ECM fibrillar proteins for growth factors led to a combinational approach for optimal immobilization and delivery of factors. In this direction, PCL scaffolds were coated with collagen previously bound with VEGF, which resulted in efficient stimulation of angiogenesis in vivo (Ferrara et al. 2003b).

3.2.2 Materials Ornamentation with Cell-Laid ECM

Attempts have been made to mimic the natural extracellular matrix by immobilizing naturally derived biomolecules on the surface of polymer scaffolds, but this does not reconstitute the natural diversity and complexity of cell-laid ECM.

The artificial combination of scaffolding material bioactive elements is associated with inherent limits. First, it may be effective in specific scenarios in which key genes/proteins have been identified as key trigger of the biological process to be induced (e.g., VEGF or BMP-2). Second, only a limited number of factors can be combined since involving a laborious protocol.

As an alternative, cell-driven deposition of ECM on 3D structures has been proposed to enhance the biological performance of the material. The concept relies on first a production phase leading to ECM secretion/deposition at the surface of the scaffold, followed by decellularization steps for subsequent storage as off-the-shelf substrate. As opposed to their purified counterparts, cell-laid decorated ECM offers a more complex environment composed of a multitude of factors at physiological concentrations.

This approach has been first successfully implemented combining titanium material and rat mesenchymal stromal cell (MSC)-derived ECM. The ECM was deposited along the differentiation of the cells toward the osteogenic lineage which resulted in a scaffold homogenously covered with ECM (Pham et al. 2009). Following decellularization and reseeding with primary rat MSC, ECM-coated scaffold significantly enhanced their osteoblastic differentiation in vitro, as compared to "naked" titanium material.

On the same line, synthetic polymers were ornamented with ECM deposited by human MSC during 3D culture in perfusion bioreactor (Sadr et al. 2012). The resulting ECM-polymer hybrid constructs successfully recruited host progenitors forming mineralized tissue in a stringent ectopic model. However, no frank bone formation was observed. This concept currently holds important promise for bone tissue strategies.

Still in the context of skeletal tissue repair, collagen scaffold and human MSC have been successfully combined to generate in vitro hypertrophic cartilage tissue. The resulting templates were shown to directly induce endochondral formation of bone and bone marrow ectopically in mouse models, providing a suitable decellularization method (Bourgine et al. 2014a).

While these approaches hold great promises, limits still arise from the variations associated with the use of primary cell sources. One alternative may consist in the generation of cell lines, for the engineering of cell-laid matrices with standard and reproducible properties (Bourgine et al. 2014b). This has been initiated by coating ceramic material with ECM deposited by human death-inducible mesenchymal lines, which resulted in a superior bone formation in a rat cranial defect model (Bourgine et al. 2017). In the same study, the authors also proposed enriching the ECM in specific growth factors by exploiting dedicated lines overexpressing key protein of interest. This was exemplified with VEGFα, which was naturally embedded after cell secretion and retained in the functionalized scaffold following decellularization (Bourgine et al. 2017). The method could offer the possibility to

fine-tune the composition of biological ECM, thus approaching the design flexibility of their synthetic counterpart.

4 Conclusions

Nowadays materials of increasing complexity are being designed, resulting from the strategies depicted in this chapter and their eventual combination. This is actually driven by the growing demand for replacement of complex tissue and associated functions that existing 3D structures struggle to fulfill. However, significant advances have been made in fabricating bioactive grafts/scaffolds capable to trigger a desired cellular response or mechanism of regeneration. The further development of smart 3D material will rely on the synergistic evolution of (i) material, (ii) biotechnologies, and (iii) biological sciences. On the first hand, material sciences will allow the finding of additional biocompatible materials and/or their combination for optimized substrate generation. Biotechnologies will be essential for the robust and reproducible design of precise structures, including patterning by advanced 3D printing-/laser-associated technologies toward nanoscale range resolution. And finally, biology remains key in order to identify novel instructive factors but also to better understand the complex events of tissue formation, regeneration, but also inflammation. Ideally, this would offer the possibility to not only tailored tissue-specific 3D materials but also patient-specific according to their genetic background and medical history.

Finally, regenerative strategies may be subject to a paradigm shift via the attractive concept of developmental engineering (Lenas et al. 2009). In this regard, efficacy of repair may not necessarily be achieved by tissue-engineered grafts matching the composition/properties of a mature tissue. Instead, it should rather consist in templates containing the necessary set of signals, referred to as "organ germ" (Martin 2014), priming tissue formation processes similar to embryonic events.

Acknowledgments This work was supported by the AO Foundation (grant S-15-25B) and the Knut and Alice Wallenberg Foundation.

References

Ambrosio AMA, Allcock HR, Katti DS, Laurencin CT (2002) Degradable polyphosphazene/poly (α-hydroxyester) blends: degradation studies. Biomaterials. https://doi.org/10.1016/S0142-9612(01)00293-9

Anaraki NA, Rad LR, Irani M, Haririan I (2015) Fabrication of PLA/PEG/MWCNT electrospun nanofibrous scaffolds for anticancer drug delivery. J Appl Polym Sci 132. https://doi.org/10.1002/app.41286

Badylak SF (2007) The extracellular matrix as a biologic scaffold material. Biomaterials 28:3587–3593. https://doi.org/10.1016/j.biomaterials.2007.04.043

Berrier AL, Yamada KM (2007) Cell – matrix adhesion. J Cell Physiol 213:565–573. https://doi.org/10.1002/JCP

Bhati RS, Mukherjee DP, McCarthy KJ, Rogers SH, Smith DF, Shalaby SW (2001) The growth of chondrocytes into a fibronectin-coated biodegradable scaffold. J Biomed Mater Res 56:74–82. https://doi.org/10.1002/1097-4636(200107)56:1<74::AID-JBM1070>3.0.CO;2-M

Bi L, Cao Z, Hu Y, Song Y, Yu L, Yang B, Mu J, Huang Z, Han Y (2011) Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering. J Mater Sci Mater Med 22:51–62. https://doi.org/10.1007/s10856-010-4177-3

Bourgine PE, Scotti C, Pigeot S, Tchang LA, Todorov A, Martin I (2014a) Osteoinductivity of engineered cartilaginous templates devitalized by inducible apoptosis. Proc Natl Acad Sci U S A 111:17426–17431. https://doi.org/10.1073/pnas.1411975111

Bourgine P, Le Magnen C, Pigeot S, Geurts J, Scherberich A, Martin I (2014b) Combination of immortalization and inducible death strategies to generate a human mesenchymal stromal cell line with controlled survival. Stem Cell Res 12:584. https://doi.org/10.1016/j.scr.2013.12.006

Bourgine PE, Gaudiello E, Pippenger B, Jaquiery C, Klein T, Pigeot S, Todorov A, Feliciano S, Banfi A, Martin I (2017) Engineered extracellular matrices as biomaterials of tunable composition and function. Adv Funct Mater:1605486. https://doi.org/10.1002/adfm.201605486

Cao J, Sun C, Zhao H, Xiao Z, Chen B, Gao J, Zheng T, Wu W, Wu S, Wang J, Dai J (2011) The use of laminin modified linear ordered collagen scaffolds loaded with laminin-binding ciliary neurotrophic factor for sciatic nerve regeneration in rats. Biomaterials 32:3939–3948. https://doi.org/10.1016/j.biomaterials.2011.02.020

Carragee EJ, Hurwitz EL, Weiner BK (2011) A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J 11:471–491

Centola M, Abbruzzese F, Scotti C, Barbero A, Vadalà G, Denaro V, Martin I, Trombetta M, Rainer A, Marsano A (2013) Scaffold-based delivery of a clinically relevant anti-angiogenic drug promotes the formation of in vivo stable cartilage. Tissue Eng Part A 19:1960–1971. https://doi.org/10.1089/ten.TEA.2012.0455

Chen RR, Mooney DJ (2003) Polymeric growth factor delivery strategies for tissue engineering. Pharm Res 20:1103–1112

Chen Q, Thouas GA (2015) Metallic implant biomaterials. Mater Sci Eng R Rep 87:1–57

Chua CK, Leong KF, Wang Y (2005) Fabrication of customised scaffolds using computer-aided design and rapid prototyping techniques. Computer (Long Beach Calif) 4:249–259. https://doi.org/10.1108/13552540510612938

Chung HJ, Park TG (2007) Surface engineered and drug releasing pre-fabricated scaffolds for tissue engineering. Adv Drug Deliv Rev 59:249–262

Cross M, Dexter TM (1991) Growth factors in development, transformation, and tumorigenesis. Cell 64:271–280. https://doi.org/10.1016/0092-8674(91)90638-F

Depalle B, Qin Z, Shefelbine SJ, Buehler MJ (2015) Influence of cross-link structure, density and mechanical properties in the mesoscale deformation mechanisms of collagen fibrils. J Mech Behav Biomed Mater 52:1–13. https://doi.org/10.1016/j.jmbbm.2014.07.008

Dhandayuthapani B, Yoshida Y, Maekawa T, Kumar DS (2011) Polymeric scaffolds in tissue engineering application: a review. Int J Polym Sci 2011

Discher DE, Janmey P, Wang Y-L (2005) Tissue cells feel and respond to the stiffness of their substrate. Science 310:1139–1143. https://doi.org/10.1126/science.1116995

Douglas T, Haugen HJ (2008) Coating of polyurethane scaffolds with collagen: comparison of coating and cross-linking techniques. J Mater Sci Mater Med:2713–2719

Edelman ER, Mathiowitz E, Langer R, Klagsbrun M (1991) Controlled and modulated release of basic fibroblast growth factor. Biomaterials 12:619–626. https://doi.org/10.1016/0142-9612(91)90107-L

Ehrbar M, Schoenmakers R, Christen EH, Fussenegger M, Weber W (2008) Drug-sensing hydrogels for the inducible release of biopharmaceuticals. Nat Mater 7:800–804. https://doi.org/10.1038/nmat2250

Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689. https://doi.org/10.1016/j.cell.2006.06.044

Ewald CY, Landis JN, Porter Abate J, Murphy CT, Blackwell TK (2015) Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. Nature 519:97–101. https://doi.org/10.1038/nature14021

Ferrara N, Gerber H-P, LeCouter J (2003a) The biology of VEGF and its receptors. Nat Med 9:669–676. https://doi.org/10.1038/nm0603-669

Ferrara N, Gerber HP, LeCouter J (2003b) The biology of VEGF and its receptors. Nat Med 9:669–676. https://doi.org/10.1038/nm0603-669

Frantz C, Stewart KM, Weaver VM (2010) The extracellular matrix at a glance. J Cell Sci 123:4195–4200. https://doi.org/10.1242/jcs.023820

Friess W, Uludag H, Foskett S, Biron R, Sargeant C (1999) Characterization of absorbable collagen sponges as rhBMP-2 carriers. Int J Pharm 187:91–99. https://doi.org/10.1016/S0378-5173(99)00174-X

Gleeson JP, Plunkett NA, O'Brien FJ (2010) Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration. Eur Cells Mater 20:218–230. https://doi.org/10.22203/eCM.v020a18

Grayson WL, Fröhlich M, Yeager K, Bhumiratana S, Chan ME, Cannizzaro C, Wan LQ, Liu XS, Guo XE, Vunjak-Novakovic G (2010) Engineering anatomically shaped human bone grafts. Proc Natl Acad Sci U S A 107:3299–3304. https://doi.org/10.1073/pnas.0905439106

Grover CN, Gwynne JH, Pugh N, Hamaia S, Farndale RW, Best SM, Cameron RE (2012) Crosslinking and composition influence the surface properties, mechanical stiffness and cell reactivity of collagen-based films. Acta Biomater 8:3080–3090. https://doi.org/10.1016/j.actbio.2012.05.006

Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS (2009) Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 5:17–26

Guillaume O, Geven MA, Sprecher CM, Stadelmann VA, Grijpma DW, Tang TT, Qin L, Lai Y, Alini M, de Bruijn JD, Yuan H, Richards RG, Eglin D (2017) Surface-enrichment with hydroxyapatite nanoparticles in stereolithography-fabricated composite polymer scaffolds promotes bone repair. Acta Biomater. https://doi.org/10.1016/j.actbio.2017.03.006

Harley BAC, Kim H-D, Zaman MH, Yannas IV, Lauffenburger DA, Gibson LJ (2008) Microarchitecture of three-dimensional scaffolds influences cell migration behavior via junction interactions. Biophys J 95:4013–4024. https://doi.org/10.1529/biophysj.107.122598

Haugh MG, Murphy CM, McKiernan RC, Altenbuchner C, O'Brien FJ (2011) Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. Tissue Eng Part A 17:1201–1208. https://doi.org/10.1089/ten.tea.2010.0590

Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316

Hench LL (1991) Bioceramics: from concept to clinic. J Am Ceram Soc 74:1487–1510

Hollinger JO, Schmitt JM, Buck DC, Shannon R, Joh S-PP, H.D. Z, Wozney J, Zegzula HD (1998) Recombinant human bone morphogenetic protein-2 and collagen for bone regeneration. J Biomed Mater Res 43:356–364

Huang M-H, Li S, Hutmacher DW, Schantz J-T, Vacanti CA, Braud C, Vert M (2004) Degradation and cell culture studies on block copolymers prepared by ring opening polymerization of ε-caprolactone in the presence of poly(ethylene glycol). J Biomed Mater Res A 69:417–427. https://doi.org/10.1002/jbm.a.30008

Hutmacher DW, Schantz JT, Lam CXF, Tan KC, Lim TC (2007) State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. J Tissue Eng Regen Med 1:245–260. https://doi.org/10.1002/term.24

Inzana JA, Olvera D, Fuller SM, Kelly JP, Graeve OA, Schwarz EM, Kates SL, Awad HA (2014) 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. Biomaterials 35:4026–4034. https://doi.org/10.1016/j.biomaterials.2014.01.064

Katti DS, Lakshmi S, Langer R, Laurencin CT (2002) Toxicity, biodegradation and elimination of polyanhydrides. Adv Drug Deliv Rev 54:933–961

Lee KY, Peters MC, Anderson KW, Mooney DJ (2000) Controlled growth factor release from synthetic extracellular matrices. Nature 408:998–1000. https://doi.org/10.1038/35050141

LeGeros RZ (2008) Calcium phosphate-based osteoinductive materials. Chem Rev 108:4742–4753

Lenas P, Moos M, Luyten FP (2009) Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part I: from three-dimensional cell growth to biomimetics of in vivo development. Tissue Eng Part B Rev 15:381–394. https://doi.org/10.1089/ten.TEB.2008.0575

Lukjanenko L, Jung MJ, Hegde N, Perruisseau-Carrier C, Migliavacca E, Rozo M, Karaz S, Jacot G, Schmidt M, Li L, Metairon S, Raymond F, Lee U, Sizzano F, Wilson DH, Dumont NA, Palini A, Fässler R, Steiner P, Descombes P, Rudnicki MA, Fan C-M, von Maltzahn J, Feige JN, Bentzinger CF (2016) Loss of fibronectin from the aged stem cell niche affects the regenerative capacity of skeletal muscle in mice. Nat Med. https://doi.org/10.1038/nm.4126

Lutolf MP, Hubbell J a (2005) Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 23:47–55. https://doi.org/10.1038/nbt1055

Lutolf MP, Weber FE, Schmoekel HG, Schense JC, Kohler T, Muller R, Hubbell JA (2003) Repair of bone defects using synthetic mimetics of collagenous extracellular matrices. Nat Biotechnol 21:513–518. https://doi.org/10.1038/nbt818

Martin I (2014) Engineered tissues as customized organ germs. Tissue Eng Part A 20:2013–2014. https://doi.org/10.1089/ten.tea.2013.0772

Martina M, Hutmacher DW (2007) Biodegradable polymers applied in tissue engineering research: a review. Polym Int 56:145–157. https://doi.org/10.1002/pi.2108

Matsiko A, Gleeson JP, O'Brien FJ (2014) Scaffold mean pore size influences mesenchymal stem cell chondrogenic differentiation and matrix deposition. Tissue Eng Part A 00:1–12. https://doi.org/10.1089/ten.TEA.2013.0545

Maynard HD, Hubbell JA (2005) Discovery of a sulfated tetrapeptide that binds to vascular endothelial growth factor. Acta Biomater 1:451–459. https://doi.org/10.1016/j.actbio.2005.04.004

Melchels FPW, Tonnarelli B, Olivares AL, Martin I, Lacroix D, Feijen J, Wendt DJ, Grijpma DW (2011) The influence of the scaffold design on the distribution of adhering cells after perfusion cell seeding. Biomaterials 32:2878–2884. https://doi.org/10.1016/j.biomaterials.2011.01.023

Murphy SV, Atala A (2014) 3D bioprinting of tissues and organs. Nat Biotechnol 32:773–785. https://doi.org/10.1038/nbt.2958

Murphy WL, Peters MC, Kohn DH, Mooney DJ (2000) Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. Biomaterials 21:2521–2527. https://doi.org/10.1016/S0142-9612(00)00120-4

Murphy CM, Matsiko A, Haugh MG, Gleeson JP, O'Brien FJ (2012) Mesenchymal stem cell fate is regulated by the composition and mechanical properties of collagen-glycosaminoglycan scaffolds. J Mech Behav Biomed Mater 11:53–62. https://doi.org/10.1016/j.jmbbm.2011.11.009

Ovsianikov A, Yoo J, Mironov V (2018) 3D printing and biofabrication. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-45444-3

Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM (2004) Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. J Clin Invest 113:516–527. https://doi.org/10.1172/JCI200418420

Park JY, Shim J-H, Choi S-A, Jang J, Kim M, Lee SH, Cho D-W (2015) 3D printing technology to control BMP-2 and VEGF delivery spatially and temporally to promote large-volume bone regeneration. J Mater Chem B 3:5415–5425. https://doi.org/10.1039/C5TB00637F

Pham QP, Kasper FK, Mistry AS, Sharma U, Yasko AW, Jansen JA, Mikos AG (2009) Analysis of the osteoinductive capacity and angiogenicity of an in vitro generated extracellular matrix. J Biomed Mater Res Part A 88:295–303. https://doi.org/10.1002/jbm.a.31875

Reddi AH (1998) Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 16:247–252. https://doi.org/10.1038/nbt0398-247

Richardson TP, Peters MC, Ennett AB, Mooney DJ (2001) Polymeric system for dual growth factor delivery. Nat Biotechnol 19:1029–1034. https://doi.org/10.1038/nbt1101-1029

Rodrigues CVM, Serricella P, Linhares ABR, Guerdes RM, Borojevic R, Rossi MA, Duarte MEL, Farina M (2003) Characterization of a bovine collagen-hydroxyapatite composite scaffold for bone tissue engineering. Biomaterials 24:4987–4997. https://doi.org/10.1016/S0142-9612(03)00410-1

Roehlecke C, Witt M, Kasper M, Schulze E, Wolf C, Hofer A, Funk RHW (2001) Synergistic effect of titanium alloy and collagen type I on cell adhesion, proliferation and differentiation of osteoblast-like cells. Cells Tissues Organs 168:178–187. https://doi.org/10.1159/000047833

Roohani-Esfahani S-I, Newman P, Zreiqat H (2016) Design and fabrication of 3D printed scaffolds with a mechanical strength comparable to cortical bone to repair large bone defects. Sci Rep 6:19468. https://doi.org/10.1038/srep19468

Sacchi V, Mittermayr R, Hartinger J, Martino MM, Lorentz KM, Wolbank S, Hofmann A, Largo RA, Marschall JS, Groppa E, Gianni-Barrera R, Ehrbar M, Hubbell JA, Redl H, Banfi A (2014) Long-lasting fibrin matrices ensure stable and functional angiogenesis by highly tunable, sustained delivery of recombinant VEGF164. Proc Natl Acad Sci 111:6952–6957. https://doi.org/10.1073/pnas.1404605111

Sadr N, Pippenger BE, Scherberich A, Wendt D, Mantero S, Martin I, Papadimitropoulos A (2012) Enhancing the biological performance of synthetic polymeric materials by decoration with engineered, decellularized extracellular matrix. Biomaterials 33:5085–5093. https://doi.org/10.1016/j.biomaterials.2012.03.082

Saif J, Schwarz TM, Chau DYS, Henstock J, Sami P, Leicht SF, Hermann PC, Alcala S, Mulero F, Shakesheff KM, Heeschen C, Aicher A (2010) Combination of injectable multiple growth factor-releasing scaffolds and cell therapy as an advanced modality to enhance tissue neovascularization. Arterioscler Thromb Vasc Biol 30:1897–1904. https://doi.org/10.1161/ATVBAHA.110.207928

Sannino A, Madaghiele M (2009) Tuning the porosity of collagen-based scaffolds for use as nerve regenerative templates. J Cell Plast 45:137–155. https://doi.org/10.1177/0021955X08099930

Shea LD, Smiley E, Bonadio J, Mooney DJ (1999) DNA delivery from polymer matrices for tissue engineering. Nat Biotechnol 17:551–554. https://doi.org/10.1038/9853

Shen W, Chen X, Hu Y, Yin Z, Zhu T, Hu J, Chen J, Zheng Z, Zhang W, Ran J, Heng BC, Ji J, Chen W, Ouyang HW (2014) Long-term effects of knitted silk-collagen sponge scaffold on anterior cruciate ligament reconstruction and osteoarthritis prevention. Biomaterials 35:8154–8163. https://doi.org/10.1016/j.biomaterials.2014.06.019

Sheridan MH, Shea LD, Peters MC, Mooney DJ (2000) Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery. J Control Release 64:91–102

Suárez-González D, Lee JS, Diggs A, Lu Y, Nemke B, Markel M, Hollister SJ, Murphy WL (2014) Controlled multiple growth factor delivery from bone tissue engineering scaffolds via designed affinity. Tissue Eng Part A 20:2077–2087. https://doi.org/10.1089/ten.tea.2013.0358

Tian Y, Jiang X, Chen X, Shao Z, Yang W (2014) Doxorubicin-loaded magnetic silk fibroin nanoparticles for targeted therapy of multidrug-resistant cancer. Adv Mater 26:7393–7398. https://doi.org/10.1002/adma.201403562

Toni R, Tampieri A, Zini N, Strusi V, Sandri M, Dallatana D, Spaletta G, Bassoli E, Gatto A, Ferrari A, Martin I (2011) Ex situ bioengineering of bioartificial endocrine glands: a new frontier in regenerative medicine of soft tissue organs. Ann Anat 193:381–394

Wang Q, Li H, Xiao Y, Li S, Li B, Zhao X, Ye L, Guo B, Chen X, Ding Y, Bao C (2015) Locally controlled delivery of TNF?? Antibody from a novel glucose-sensitive scaffold enhances alveolar bone healing in diabetic conditions. J Control Release 206:232–242. https://doi.org/10.1016/j.jconrel.2015.03.019

Williams DF (2009) On the nature of biomaterials. Biomaterials 30:5897–5909. https://doi.org/10.1016/j.biomaterials.2009.07.027

Wojtowicz AM, Shekaran A, Oest ME, Dupont KM, Templeman KL, Hutmacher DW, Guldberg RE, García AJ (2010) Coating of biomaterial scaffolds with the collagen-mimetic peptide

GFOGER for bone defect repair. Biomaterials 31:2574–2582. https://doi.org/10.1016/j.biomaterials.2009.12.008

Yoo HS, Kim TG, Park TG (2009) Surface-functionalized electrospun nanofibers for tissue engineering and drug delivery. Adv Drug Deliv Rev 61:1033–1042

Zhan Q, Shen B, Deng X, Chen H, Jin J, Zhang X, Peng C, Li H (2013) Drug-eluting scaffold to deliver chemotherapeutic medication for management of pancreatic cancer after surgery. Int J Nanomedicine 8:2465–2472. https://doi.org/10.2147/IJN.S47666

Zheng L, Yang F, Shen H, Hu X, Mochizuki C, Sato M, Wang S, Zhang Y (2011) The effect of composition of calcium phosphate composite scaffolds on the formation of tooth tissue from human dental pulp stem cells. Biomaterials 32:7053–7059. https://doi.org/10.1016/j.biomaterials.2011.06.004

Peeking into a Hidden Syndicate: Mitochondria of the Human Amniotic Membrane

Adelheid Weidinger and Asmita Banerjee

Contents

Abstract

Despite great leaps in tissue engineering development, the human amniotic membrane (hAM), a natural biomaterial, is still in the race. The discovery that cells of the hAM possess stem cell characteristics created new options for the clinical use of hAM for tissue regeneration. Furthermore, cell organelles, such as mitochondria, have recently been recognized to be involved in regenerative processes. Mitochondria cover various functions and play an important role in many cellular death/rescue programs. The aim of this review is to show how far research has come on this very connection with regard to the hAM. Studies show that mitochondria-related processes such as senescence, apoptosis, and autophagy in the hAM seem to contribute to rupture of membranes and human parturition. Although many of these factors indicate a process similar to acute inflammation, their exact interplay is not well understood. With regard to tissue regeneration, research on mitochondrial interactions in the hAM is still in its infancy. All the more it appears important to focus on this issue in future studies, which will

A. Weidinger (✉) · A. Banerjee
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center, Vienna, Austria
e-mail: adelheid.weidinger@trauma.lbg.ac.at; asmita.banerjee@trauma.lbg.ac.at

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_64

further help to evaluate the quality of hAM and its suitability for tissue engineering.

Abbreviations

ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
BCL-2	B-cell lymphoma 2
ETS	Electron transfer system
hAECs	Human amniotic epithelial cells
hAM	Human amniotic membrane
hAMSCs	Human amniotic mesenchymal stromal cells
MnSOD	Manganese superoxide dismutase
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
Nrf2	Nuclear respiratory factor 2
PPAR	Peroxisome proliferator-activated receptors
PROM	Premature rupture of membrane
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
TCA	Tricarboxylic acid
TMRM	Tetramethylrhodamine methyl ester
TNF	Tumor necrosis factor

1 Introduction

In 1910, J. S. Davis first reported the potential surgical use of the human amniotic membrane (hAM), the innermost of the fetal membranes, as skin coverage material for wounds (Davis 1910). Although Davis himself did not achieve favorable results, he stated, ". . .if a technic is developed by which favorable results can be obtained, it may be of great use" (Davis 1910). Within a few years, the hAM indeed proved to be of great use for a number of clinical applications, including coverage for wound healing, burn injuries, tissue reconstruction, and ophthalmology (Stern 1912; Sabella 1913; Brindeau 1934; De Rötth 1940). In general, the hAM is devitalized or denuded prior to clinical application, taking advantage of the extracellular matrix enclosing secreted factors, left behind from resident cells.

With the turn of the millennium, researchers started to understand that cells of the hAM, human amniotic epithelial cells (hAECs) and human amniotic mesenchymal stromal cells (hAMSCs), display distinct stem cell characteristics. This includes the expression of pluripotency markers and mesenchymal stem cell markers and the ability of the cells to differentiate into lineages of all three germ layers in vitro and in vivo. With this insight gained, the option of using vital hAM has to be considered for application in tissue regeneration in a clinical context.

The use of *vital* hAM, containing extracellular matrix and vital cells, reshuffled the cards, and all of a sudden, a bunch of bustling cell organelles participated in the game. Among the many fascinating organelles of a cell, mitochondria have recently moved into the focus of research. For a long time, mitochondria were regarded as mere producers of "bioenergy," and only recent research illustrated their importance as signaling organelles (reviewed in (Galluzzi et al. 2012)), especially in regard to stem cells (reviewed in (Oh et al. 2014)). Now mitochondrial function and cell fate, intermingling at multiple checkpoints, can no longer be dealt with separately. The aim of this review is to show how far research has come on this very connection with regard to the hAM (Table 1).

2 Morphology

The search for mitochondria in the hAM started with L.A. Emge as early as 1921 (Emge 1921). Emge found mitochondria in the hAM at all stages of gestation. The period of gestation did not show any influence on type, shape, size, number, and arrangement of mitochondria, except in the earliest stages of gestation. The mitochondrial arrangement in the hAM correlated with the shape of the cells and cell type. Of note, although the hAM was fixed, cells of the hAM had a poor affinity for dyes. Remarkably, Emge already observed that different areas of the hAM showed different mitochondrial appearances. Therefore, for sample preparation, the exact locations of hAM specimens were carefully documented. At term, Emge observed greatest uniformity in size and shape of mitochondria in the inner two-thirds of the placenta and highly heterogeneous appearances in reflected amnion, the amnion that does not cover the placenta. This was the first indication for region-dependent mitochondrial differences in the hAM.

Nevertheless, most studies on mitochondrial morphology in the hAM do not consider the difference between amniotic regions, but rather focus on different layers of the hAM. The majority of reports investigate the innermost layer of the hAM, the amniotic epithelium. C. E. Thomas stated that the cells of the amniotic epithelium could be divided into two distinct cell populations (Thomas 1965). The "Golgi type," with highly developed Golgi apparatuses, and mitochondria distributed throughout the cell, however, sometimes located specifically in the basal and paranuclear areas. The second cell type, the "fibrillar type," has smaller Golgi apparatuses and high contents of coarse fibrils in the cytoplasm. Mitochondria of the fibrillar type of cells are more electron-dense, coarsely granular, and fewer in number, compared to the Golgi type. With these observations, Thomas discussed a possible secretory function by the Golgi-type cell. Regarding cell morphology, Thomas described differences in placental and reflected amnion, however, with both aforementioned cell types present. This study included hAMs of cesarean sections and normal deliveries.

The cellular heterogeneity of the amniotic epithelium has been a matter of controversy, ever since. Addressing the question, whether the cells of the amniotic epithelium are homogeneous or heterogeneous, Iwasaki and Matsubara investigated

Table 1 Literature review summary table: measurement and analysis of mitochondria in the human amniotic membrane. Abbreviations: *ATP* adenosine triphosphate, *hAEC* human amniotic epithelial cells, *hAM* human amniotic membrane, *hAMSC* human amniotic mesenchymal stromal cells, *MnSOD* manganese-dependent superoxide dismutase, *mtDNA* mitochondrial DNA, *OXPHOS* oxidative phosphorylation, and *SDH* succinate dehydrogenase

Mitochondrial morphology, number, localization, and appearance		**Author**
Gestation stage-dependent morphology	In the earliest stages of gestation, mitochondrial morphology different to other periods of gestation	Emge 1921
Region-specific morphology	Region-specific mitochondrial morphology of hAM	Emge 1921
Cell type-specific morphology	Different morphology in two cell types of hAECs	Thomas 1965
	Long and filamentous in hAMSCs, fragmented in hAECs	Banerjee et al. 2015, 2018a
Cell type-specific number, electron density, and localization	Different number, electron density, and localization in two cell types of hAECs ("fibrillar" and "Golgi" type)	Thomas 1965
Infection-induced alterations in morphology, number, and appearance	Time-dependent alterations after skin-virus suspension challenge	Burnett and Sutton 1968
Cell sub-type-dependent membrane potential	At least two cell types differ in mitochondrial membrane potential in epithelial layer	Banerjee et al. 2015
Cell environment-induced changes	Type of storage solution changed mitochondrial density	Hoyes 1972
Senescence, apoptosis, and autophagy		**Author**
Senescence in labor at term	Mitochondrial senescence marker in labor at term	Behnia et al. 2015
	Swollen mitochondria and vacuolization in labor at term	Liu et al. 2017
Apoptosis in preterm/term conditions	No involvement of mitochondria-mediated pathway	Kumagai et al. 2001; Sağol et al. 2002
	Involvement of mitochondria-mediated pathway	Menon et al. 2002; Fortunato et al. 2000; Shen et al. 2008a, b
Autophagy at term	Transcription of beclin 1	Shen et al. 2008a
	Autophagic vacuoles near ruptured areas	Shen et al. 2008b
Mitochondrial reactive oxygen species and antioxidants		**Author**
Region-specific ROS levels	Lower ROS levels in placental amnion biopsies and hAMSCs	Banerjee et al. 2015, 2018a
Cell type-specific ROS levels	Higher ROS levels in hAECs versus hAMSCs	Banerjee et al. 2018a
Stress-induced ROS levels	Cigarette smoke extract-induced increase in ROS levels	Polettini et al. 2014
ROS signaling → Ca^{2+} metabolism	Superoxide increased Ca^{2+} concentrations in hAECs	Ikebuchi et al. 1991

(continued)

Table 1 (continued)

ROS signaling → MMP9 activity	Superoxide increased MMP9 activity in hAM	Buhimischi et al. 2000
MnSOD involvement in term and preterm labor	No involvement	Telfer et al. 1997
	Involvement	Than et al. 2009
Metabolism		**Author**
Oxygen dependence of ATP production	Mainly anaerobic metabolism in amniotic epithelium	Benedetti et al. 1973
	Low OXPHOS activity in isolated hAMSCs and hAECs	Banerjee et al. 2018a
	Oxygen-dependent switch from glycolysis to OXPHOS	Banerjee et al. 2018b
Cell-specific mitochondrial respiratory activity	Level of succinate dehydrogenase in amniotic epithelium higher compared to amniotic mesoderm	Weser and Kaufmann 1978
Region-specific mitochondrial respiratory activity	Higher levels of SDH in placental versus reflected amnion	Banerjee et. al. 2018a
	Higher respiration in placental versus reflected biopsies	Banerjee et al. 2015
	Higher respiration in placental hAECs and hAMSCs versus reflected cells	Banerjee et al. 2018a
Mitochondrial DNA		**Author**
Region-specific differences	Higher mtDNA copy number in reflected versus placental hAM	Banerjee et al. 2018a
Cell type-specific differences	Higher mtDNA copy number in hAECs versus hAMSCs	Banerjee et al. 2018a

cellular parameters linked to mitochondria and other cell organelles. In the first study, they traced cytochrome c oxidase in mitochondria and non-mitochondrial enzymes such as alkaline phosphatase, glucose-6-phosphatase, glucose-6-phosphatase dehydrogenase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in cellular structures such as lysosomes and plasma membranes (Iwasaki et al. 2003). The enzyme distribution pattern appeared similar throughout the amniotic epithelium, as did the morphology of mitochondria and other cell organelles, indicating a homogeneous, rather than heterogeneous cell population of the amniotic epithelium (Iwasaki et al. 2003). In contrast, another study revealed two different cell populations with distinguished electron density or darkness of cytoplasm, but with no differences in structure and number of mitochondria and Golgi apparatus (Matsubara and Iwasaki 2004). Both studies were performed with reflected amnion of cesarean sections (Iwasaki et al. 2003; Matsubara and Iwasaki 2004).

The suggestion that (at least) two different cell populations exist in the amniotic epithelium is in line with results from our group (Banerjee et al. 2015). Although we did not detect differences in mitochondrial morphology within the amniotic epithelium, we found mitochondria with different mitochondrial membrane potential in biopsies (Banerjee et al. 2015) and isolated cells (Banerjee et al. 2018a). Of note, under atmospheric oxygen, single cells with low mitochondrial membrane potential (stained with MitoTracker™ Green) were found scattered all over the epithelium,

surrounded by cells with high mitochondrial membrane potential (stained with TMRM) (Banerjee et al. 2015).

Regarding mitochondrial morphology, very little data is available on hAMSCs of the hAM, also referred to as fibroblasts in some reports. Banerjee et al. found mitochondria in hAMSCs to be predominantly long and filamentous (Banerjee et al. 2015, 2018a). Such mitochondrial morphology was also found by Essner and colleagues (1965). However, although not further elaborated, the method for cell isolation in this study clearly suggests cultures dominated by hAECs, rather than hAMSCs. This discrepancy can be explained by the fact that mitochondria adapt their morphology to changes in the cellular environment to maintain functional mitochondria (reviewed in (Chen and Chan 2009; Galloway et al. 2012; Youle and van der Bliek 2012)). Accordingly, Edwards and Fogh already showed that treatment of amniotic cells with trypsin leads to hypertrophy of mitochondria, which is considered as defense mechanisms for cell survival (Edwards and Fogh 1959).

Similar alterations in morphology of mitochondria were shown in cultured amniotic cells challenged with skin-virus suspension (*Molluscum contagiosum*) (Burnett and Sutton 1968). The cell species was not further specified, but the cell isolation method with trypsin indicated that the cells were mainly hAECs. Electron microscopy revealed a variety of alterations in mitochondrial morphology. Early alterations (24 h) appeared as a reduction in number of mitochondrial cristae. After 48 h, condensed mitochondria with irregular matrix and myelin-figure arrangement of membranes were found (Burnett and Sutton 1968). A. D. Hoyes found increased density of mitochondrial matrix due to short-term storage of hAM in different storage solutions. Interestingly, Ringer's lactate solution was found to be superior to other storage solutions, such as Hanks' solution (Hoyes 1972). With regard to the storage conditions, specific oxygen levels have been shown to significantly impact cellular energy production in hAMSCs by switching glycolysis to mitochondrial oxidative phosphorylation (Banerjee et al. 2018b). These data impressively show the high sensitivity of mitochondria to changes of their milieu.

Alterations in mitochondrial morphology can also be an indication for the onset of apoptosis, senescence, and autophagy (reviewed in (Seo et al. 2010)). It is commonly accepted that mitochondria play an important part in the regulation of senescence (reviewed in (Correia-Melo and Passos 2015)), apoptosis (reviewed in (Redza-Dutordoir and Averill-Bates 2016)), and autophagy (reviewed in (Okamoto and Kondo-Okamoto 2012; Hsu and Shi 2017)).

3 Senescence, Apoptosis, and Autophagy

Many of the factors that induce one of the most incisive occurrences in human life, childbirth, are not yet fully understood. Considering the stringent timeline of gestation, it is obvious that fetal membranes are subject to a conclusive expiry date. However, does this limited life span actually play a role in processes such as parturition or preterm birth?

Biochemical and morphological changes due to cellular senescence of chorioamniotic membranes showed higher incidence of senescence in women with labor at term (spontaneous vaginal delivery) compared to women with cesarean sections (Behnia et al. 2015). Further, swollen endoplasmic reticulum and mitochondria were also found in hAM at term with labor, compared to cesarean sections. Mitochondria of labor at term also showed vacuolization, which typically occur under stress (Liu et al. 2017).

Amnion apoptosis is an important programmed process of fetal membrane remodeling leading to its rupture. Likewise, increased levels of apoptosis mediators were found with labor at term compared to cesarean sections (Hsu et al. 2000). In general, two major pathways lead to apoptotic cell death, the extrinsic death receptor pathway via Fas ligand binding and caspase 8 activation and the intrinsic mitochondrial pathway via cytochrome c release into the cytosol and subsequent caspase 9 activation (reviewed in Elmore (2007)). The mitochondrial pathway with cytochrome c release is triggered by intracellular threats such as DNA damage or by extracellular insults, leading to upregulation of B-cell lymphoma 2-associated X protein (BAX). Cytochrome c release is inhibited by mitochondrial B-cell lymphoma 2 (BCL2) protein (reviewed in (Elmore 2007)).

Regarding preterm and term conditions, contradictory data exist on whether the Fas ligand-mediated or the mitochondria-mediated pathway of apoptosis actually contributes to rupture and premature rupture of membranes (PROM). Kumagai et al. investigated the contribution of these two apoptotic pathways in hAM at term and concluded that apoptosis in the amniotic epithelium seems to play a crucial role for PROM, but is BCL2 independent (Kumagai et al. 2001). This is in line with a study of Sağol et al., who compared hAMs of normal pregnancies to PROM regarding anti-apoptotic BCL2 and pro-apoptotic BAX gene products (Sagol et al. 2002). Although changes in apoptosis mediators seem to play a role for PROM, it was not mediated by *BCL2* and *BAX* genes. Similar results were observed after cortisol stimulation of amniotic epithelium. In vitro, cortisol induced Fas-mediated apoptosis in hAEC via regulation of tissue-type plasminogen activator/plasmin system (Wang et al. 2016). Consistent with this, high levels of cortisol, cleaved-caspase-3 and tissue-type plasminogen activator were found in hAM of term with labor with spontaneous rupture of membranes (Wang et al. 2016). Wang and colleagues therefore advocate a possible connection to the action of cortisol to rupture of the membranes via activation of tissue-type plasminogen activator/plasmin system. Although BAX and BCL2 were ruled out to be involved in apoptosis by cortisol, Wang et al. stated that other members of the BCL2 family have not been investigated in this study. In addition, Menon et al. found tumor necrosis factor (TNF)-alpha-induced upregulation of caspase 8 and caspase 9 mRNA expression (Menon et al. 2002), suggesting involvement of both the mitochondria-linked apoptosis pathway via caspase 9 (Chinnaiyan 1999) and the death receptor family-linked apoptosis pathway via caspase 8 (Guicciardi and Gores 2009).

Other studies favor the mitochondria-mediated pathway. Fortunato et al. found p53 gene products elevated at PROM (Fortunato et al. 2000) which can activate metalloproteinase 2 expression (Bian and Sun 1997). Metalloproteinase 2, in turn,

triggers apoptosis by upregulation of pro-apoptotic BAX and downregulation of anti-apoptotic BCL2 (Mohammad and Kowluru 2010). Accordingly, Fortunato et al. found elevated levels of BAX gene products at PROM compared to term deliveries with labor and preterm deliveries with intact membranes (Fortunato et al. 2000). This is in line with studies of Shen and colleagues (Shen et al. 2008a, b) who, in addition to apoptosis in rupture of membranes, also investigated the involvement of autophagy. This is interesting as it has been shown that mitochondria can also act as inducers of autophagy (reviewed in (Okamoto and Kondo-Okamoto 2012)), another mechanism that can participate in programmed cell death (Galluzzi et al. 2015). Autophagic vacuoles and autophagosomes were found in hAECs of amnion at term (Shen et al. 2008b). Additionally, hAECs showed high endocytotic activity with formation of vesicles and endosomes. Shen and colleagues also observed lysosomal degradation of endosomes and autophagosomes and stated that, occasionally, autophagic and endocytotic pathways converge. Therefore, autophagy and apoptosis both occur in hAM at term (Shen et al. 2008b), but it remains unclear whether there is a connection between the two types of cell death. This matter was addressed in a study by Shen and colleagues (2008a). Near the ruptured membranes, higher occurrence of autophagic cell death and apoptotic cell death was found. Furthermore, BAX expression (pro-apoptotic) was found to be increased, and BCL2 expression (anti-apoptotic) was decreased. Thus, these results favor the intrinsic pathway, indicating mitochondria to be involved in rupture of membranes (Shen et al. 2008a).

The mitochondria-mediated apoptosis pathway (reviewed in (Redza-Dutordoir and Averill-Bates 2016)) as well as autophagy (reviewed in (Scherz-Shouval and Elazar 2007)) can be triggered by reactive oxygen species (ROS). This is not surprising, considering the fact that mitochondria, by generation of ATP, simultaneously produce ROS (reviewed in (Andreyev et al. 2005, 2015)).

4 Reactive Oxygen Species

Under physiological conditions, mitochondria are the principal ROS producers in eukaryotic cells. Beside numerous sites in mitochondria (reviewed in (Andreyev et al. 2005, 2015)), inducible ROS producers such as NADPH oxidase (NOX) also contribute to ROS production (reviewed in (Lambeth 2004)). Intriguingly, both ROS producers can further activate each other via ROS (Dikalov et al. 2012; Kröller-Schön et al. 2014) (reviewed in Weidinger and Kozlov (2015)) or Nrf2 (Kovac et al. 2015).

The levels of intracellular ROS were studied by application of CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine), a cell permeable spin probe, in biopsies (Banerjee et al. 2015) and isolated cells (Banerjee et al. 2018a) of placental and reflected region of the hAM. Electron paramagnetic resonance measurements showed lower levels of intracellular ROS and higher levels of extracellular ROS in placental compared to reflected amnion (Banerjee et al. 2015, 2018a). These observations were confirmed in isolated hAMSCs, whereas isolated hAECs did not show

sub-regional differences (Banerjee et al. 2018a). Investigation of NOX gene expression (1–5) in the fetal membranes showed NOX2 and NOX3 expression in the hAM (Polettini et al. 2014). This was especially the case with preterm premature rupture of membrane complications (Polettini et al. 2014). Absence of any changes in NOXs expression after stimulation with cigarette smoke extract suggested that ROS generation in the membranes does not always correlate with NOX expression. Notably, these ROS were of mitochondrial origin (Polettini et al. 2014).

Ahead of their time, Ikebuchi and colleagues explored the signaling effects of superoxide anion on hAECs (Ikebuchi et al. 1991). The presence of superoxide anion increased Ca^{2+} concentrations and induced the release of arachidonate, a precursor of prostaglandins. Furthermore, the induction of arachidonate release was inhibited by a decrease in extracellular Ca^{2+} concentration, suggesting that superoxide anion may regulate cellular functions in the hAM via Ca^{2+}-dependent pathways (Ikebuchi et al. 1991). Today, there is plenty of evidence that mitochondria control intracellular Ca^{2+} signaling and cell survival (reviewed in (Rizzuto et al. 2012)) via activation of pore-forming mitochondrial F-ATP synthase (Bernardi et al. 2015), which enables the release of mitochondrial ROS into the cytoplasm (reviewed in (Bernardi et al. 2015)).

Superoxide anion in human fetal membranes was also focus of a study of Buhimschi et al. (2000). In cesarean sections at term, Buhimschi and colleagues detected basal matrix metalloproteinase 2 and matrix metalloproteinase 9, which are relevant triggers of apoptosis (Mohammad and Kowluru 2010). Superoxide anion increased matrix metalloproteinase 9 but not matrix metalloproteinase 2 activity. Notably, addition of *N*-acetyl cysteine, a glutathione precursor, inhibited amniochorionic matrix metalloproteinase activity, suggesting redox regulation of matrix metalloproteinase activity in hAM. Buhimschi and colleagues therefore stated that *N*-acetyl cysteine may protect against preterm premature rupture of the membranes.

Another anti-oxidative strategy, performed by mitochondrial manganese superoxide dismutase (MnSOD), is upregulated upon inflammation and acts as an important anti-inflammatory enzyme (reviewed in (Li and Zhou 2011)). Although MnSOD was detected in the hAM of term labor, no differences in enzyme activity were found before and after the onset of labor at term (Telfer et al. 1997). Therefore, Telfer and colleagues stated that this enzyme does not seem to play a role for parturition (Telfer et al. 1997). In contrast, increased MnSOD mRNA expression was found in the hAM of term labor and preterm labor complications, possibly indicating an involvement of inflammatory processes (Than et al. 2009).

Taken together, various mitochondria-related factors such as senescence, apoptosis, autophagy, and ROS in the hAM seem to contribute to the rupture of membranes and human parturition. Their exact interplay, however, remains to be determined. Many of these factors indicate a process similar to acute inflammation. Therefore, it is not surprising that regulation of human parturition has also been linked to peroxisome proliferator-activated receptors (PPARs), an important modulator of the inflammatory response (reviewed in (Delerive et al. 2001)). PPAR alpha and gamma were found in the amniotic epithelium (Lindström and Bennett 2005), and

higher amounts of PPAR alpha, delta, and gamma were found in the amniotic epithelium of cesarean section compared to term labor placenta (Holdsworth-Carson et al. 2009). It is important to note that PPARs are also regulators of mitochondrial oxidative metabolism (reviewed in (Fan and Evans 2015)), and here again, although not obvious at first glance, we cannot escape the influence of mitochondria.

5 Metabolism

Mitochondria still have more to offer. Next to all their fancy signaling functions, generation of ATP appears almost a bit old-fashioned. Yet, stepping closer, this is no less important, since energy metabolism enables cells to fulfill their mission. Mitochondria interfere with almost all metabolic pathways including protein, carbohydrate, and lipid metabolisms. Considering the immensely important role of energy production for vital cell function, surprisingly few studies have been performed with the hAM in this regard.

First, mitochondrial adenosine triphosphatase activity was demonstrated in cultured "amniotic cells," stained with Wachstein-Meisel method (Essner et al. 1965). Unfortunately, no information was provided on cultivation period and passage numbers. Electron microscopy showed deposition of enzyme reaction product in distinct bands across the width of the mitochondria, but the exact location of deposition within the mitochondria remained unsettled.

In 1973, Benedetti et al. performed a histochemical study and found succinic dehydrogenase and lactic dehydrogenase in the amniotic epithelium (Benedetti et al. 1973). From the latter, higher levels of "slow-moving" isoenzymes were found compared to levels of "fast-moving" isoenzymes, suggesting generation of energy in the amniotic epithelium prevailed by anaerobic metabolism (Benedetti et al. 1973). This suggestion is supported by a recent study with freshly isolated cells of the hAM where epithelial and mesenchymal cells show low oxidative phosphorylation activity (Banerjee et al. 2018a). As mentioned before, it is important to note that changing the oxygen level from 5% (which is physiological for the amnion cells) to 20% (common cell culture conditions) causes a switch from glycolysis to oxidative phosphorylation (Banerjee et al. 2018b).

A number of enzymes in the hAM, including succinate dehydrogenase (SDH) were determined. SDH is an enzyme of the tricarboxylic acid (TCA) cycle and also a component of the mitochondrial respiratory chain (Weser and Kaufmann 1978). The enzymes found in the amniotic epithelium suggested high mitochondrial content and, moreover, secretory activity of the cells, whereas the amniotic mesoderm displayed enzymes of intermediary metabolism. Regarding regional differences, higher levels of SDH in placental amnion were shown by means of electron paramagnetic resonance measurement (Banerjee et al. 2018a).

Most of the studies of mitochondrial metabolism were carried out without consideration of different amniotic regions, such as placental and reflected amnion. Such a study on different amniotic regions was performed by our group. We found fourfold higher mitochondrial activity (routine respiration and maximum electron

transfer system (ETS) capacity) concomitant with higher levels of ATP in biopsies of placental amnion compared to reflected amnion (Banerjee et al. 2015). Also confocal microscopy images showed higher membrane potential of mitochondria in biopsies of the placental amnion (Banerjee et al. 2015). In freshly isolated hAECs and hAMSCs, ROUTINE respiration, maximum ETS capacity, and phosphorylation-related respiration were higher in cells from placental region (Banerjee et al. 2018a). These results provoked the question whether the higher activity in the placental region is caused by a higher mitochondrial content per cell or higher activity of individual mitochondria. A commonly used marker for mitochondrial content is mitochondrial DNA (mtDNA) copy number per cell.

6 Mitochondrial DNA

The mitochondrial genome plays an important part in mediating cellular processes such as stem cell differentiation which is characterized by increased mtDNA copy number (St. John 2016). In a study of Vaghjiani et al., hAECs did not increase their mtDNA copy number during differentiation into hepatocyte-like cells, although gene expression data and metabolic assays pointed to successful differentiation toward hepatocyte-like cells (Vaghjiani et al. 2017). The authors explain this by higher levels of DNA methylation. DNA methylation and demethylation are regulators of mtDNA copy number and cell fate (Lee et al. 2015). It is often assumed that mtDNA copy number and number of mitochondria per cell are related, as shown in a "virtual mitochondrion" (Robin and Wong 1998). Cell processes that do not follow this observation are cellular aging (Masuyama et al. 2005) or specific phases of the cell cycle during proliferation (Trinei et al. 2006).

In freshly isolated hAM cells, measurement of mtDNA copy number revealed higher mtDNA copy number in reflected amnion compared to placental amnion in both hAECs and hAMSCs (Banerjee et al. 2018a). This demonstrates that in the amniotic membrane the cellular mtDNA copy number is tightly controlled in a region-specific manner (Banerjee et al. 2018a). Of note, mtDNA copy number in both cell types was inversely related to mitochondrial activity, possibly indicating that the cells adapt their mitochondrial activity according to their anatomical location (Banerjee et al. 2018a). Interestingly, the comparison of cellular mtDNA copy numbers across cell types showed higher levels of mtDNA copy numbers in hAECs compared to hAMSCs (Banerjee et al. 2018a).

7 Conclusions

More than a century ago, clinicians started to explore the potential of hAM for regenerative medicine. The excitement over this multifaceted biomaterial diminished a little over time. In the past two decades, however, revolutionary discoveries of cellular differentiation, multipotency, and "stemness" put basic research and clinical application of hAM into new perspective. Mitochondrial research also

experienced a renaissance, as it became increasingly evident that mitochondria directly impact virtually every aspect of cellular function. Almost inevitably, these developments led cell biology and mitochondrial research to approach each other. This became all the more important with growing evidence of the pivotal role mitochondria play for tissue regeneration and stem cell fate (Islam et al. 2013; Ahmad et al. 2014).

To outline the current state of knowledge on mitochondria in the hAM, the earliest publications focus on mitochondrial morphology. Various mitochondrial morphologies were described, depending on amniotic region, amniotic layer, or cell type, strongly influenced by apoptosis, senescence, or autophagy. These mitochondria-related cell death/rescue programs as well as ROS seem to be important for the onset of hAM degradation and rupture of membranes. These data let researchers draw a scenery strikingly similar to acute inflammation.

In sum, most studies focus on conditions leading to labor, term, or preterm. Data generated from these studies can certainly be deployed for tissue engineering purposes to a certain extent, but the exact mechanisms are still missing. Mechanisms, how mitochondria of vital cells in the hAM could influence tissue regeneration, are certainly far from being understood. Using vital hAM with functional mitochondria necessitates precise basic research in order to optimize its application as a biomaterial. Regional differences within the hAM, linkage to inflammatory processes, and mechanisms related to cell rescue are just a few examples, where research could reveal hidden transactions of the mitochondrial syndicate. Taken together, research on mitochondrial interactions in the hAM, particularly with regard to tissue regeneration, is at the beginning. Future studies should focus on mitochondrial interactions in hAM, as this will help both to understand physiological processes regulating preterm and term rupture of the hAM and to evaluate the quality of hAM and its suitability for tissue engineering.

References

Ahmad T, Mukherjee S, Pattnaik B, Kumar M, Singh S, Rehman R, Tiwari BK, Jha KA, Barhanpurkar AP, Wani MR, Roy SS, Mabalirajan U, Ghosh B, Agrawal A (2014) Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. EMBO J 33:994–1010

Andreyev A, Kushnareva Y, Starkov A (2005) Mitochondrial metabolism of reactive oxygen species. Biochemist 70:200–214

Andreyev AY, Kushnareva YE, Murphy AN, Starkov AA (2015) Mitochondrial ROS metabolism: 10 years later. Biochemist 80:517–531

Banerjee A, Weidinger A, Hofer M, Steinborn R, Lindenmair A, Hennerbichler-Lugscheider S, Eibl J, Redl H, Kozlov AV, Wolbank S (2015) Different metabolic activity in placental and reflected regions of the human amniotic membrane. Placenta 36:1329–1332

Banerjee A, Lindenmair A, Hennerbichler S, Steindorf P, Steinborn R, Kozlov AV, Redl H, Wolbank S, Weidinger A (2018a) Cellular and site-specific mitochondrial characterization of vital human amniotic membrane. Cell Transplant 27:3–11

Banerjee A, Lindenmair A, Steinborn R, Dumitrescu SD, Hennerbichler S, Kozlov AV, Redl H, Wolbank S, Weidinger A (2018b) Oxygen tension strongly influences metabolic parameters and the release of interleukin-6 of human amniotic mesenchymal stromal cells in vitro. Stem Cells Int 2018:1–11

Behnia F, Taylor BD, Woodson M, Kacerovsky M, Hawkins H, Fortunato SJ, Saade GR, Menon R (2015) Chorioamniotic membrane senescence: a signal for parturition? Am J Obstet Gynecol 213:359.e1–359.e16

Benedetti WL, Sala MA, Alvarez H (1973) Histochemical demonstration of enzymes in the umbilical cord and membranes of human term pregnancy. Eur J Obstet Gynecol Reprod Biol 3:185–189

Bernardi P, Rasola A, Forte M, Lippe G (2015) The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology. Physiol Rev 95:1111–1155

Bian JH, Sun Y (1997) Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. Mol Cell Biol 17:6330–6338

Brindeau A (1934) Creation d'un vagin artificiel A l'aide des membranes ovulaires d'un oeuf A terme. J Gynecol Obstet Biol Reprod 13:821–824

Buhimschi IA, Kramer WB, Buhimschi CS, Thompson LP, Weiner CP (2000) Reduction oxidation (redox) state regulation of matrix metalloproteinase activity in human fetal membranes. Am J Obstet Gynecol 182:458–464

Burnett JW, Sutton JS (1968) Molluscum contagiosum cytotoxicity for primary human amnion cells in vitro as studied by electron microscopy. J Invest Dermatol 50:67–84

Chen H, Chan DC (2009) Mitochondrial dynamics-fusion, fission, movement, and mitophagy-in neurodegenerative diseases. Hum Mol Genet 18:169–176

Chinnaiyan AM (1999) The apoptosome: heart and soul of the cell death machine. Neoplasia 1:5–15

Correia-Melo C, Passos JF (2015) Mitochondria: are they causal players in cellular senescence? Biochim Biophys Acta 1847:1373–1379

Davis J (1910) Skin transplantation with a review of 550 cases at the Johns Hopkins hospital. John Hopkins Med J 15:307–396

De Rötth A (1940) Plastic repair of conjunctival defects with fetal memranes. Arch Ophthalmol 23:522–525

Delerive P, Fruchart J-C, Staels B (2001) Peroxisome proliferator-activated receptors in inflammation control. J Endocrinol 169:453–459

Dikalov SI, Li W, Doughan AK, Blanco RR, Zafari AM (2012) Mitochondrial reactive oxygen species and calcium uptake regulate activation of phagocytic NADPH oxidase. Am J Phys Regul Integr Comp Phys 302:R1134–R1142

Edwards GA, Fogh J (1959) Micromorphologic changes in human amnion cells during trypsinization. Cancer Res 19:608

Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495–516

Emge LA (1921) Notes on the study of mitochondria in the human amnion. Anat Rec 22:342–351

Essner E, Fogh J, Fabrizio P (1965) Localization of mitochondrial adenosine triphosphatase activity in cultured human cells. J Histochem Cytochem 13:647–655

Fan W, Evans R (2015) PPARs and ERRs: molecular mediators of mitochondrial metabolism. Curr Opin Cell Biol 33:49–54

Fortunato SJ, Menon R, Bryant C, Lombardi SJ (2000) Programmed cell death (apoptosis) as a possible pathway to metalloproteinase activation and fetal membrane degradation in premature rupture of membranes. Am J Obstet Gynecol 182:1468–1476

Galloway CA, Lee H, Yoon Y (2012) Mitochondrial morphology – emerging role in bioenergetics. Free Radic Biol Med 53:1–11

Galluzzi L, Kepp O, Kroemer G (2012) Mitochondria: master regulators of danger signalling. Nat Rev Mol Cell Biol 13:780–788

Galluzzi L, Bravo-San Pedro JM, Vitale I, Aaronson SA, Abrams JM, Adam D, Kroemer G et al (2015) Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. Cell Death Differ 22:58–73

Guicciardi ME, Gores GJ (2009) Life and death by death receptors. FASEB J 23:1625–1637

Holdsworth-Carson SJ, Permezel M, Riley C, Rice GE, Lappas M (2009) Peroxisome proliferator-activated receptors and retinoid X receptor-alpha in term human gestational tissues: tissue specific and labour-associated changes. Placenta 30:176–186

Hoyes AD (1972) Fine structure of human amniotic epithelium following short-term preservation in vitro. J Anat 111:43–54

Hsu P, Shi Y (2017) Regulation of autophagy by mitochondrial phospholipids in health and diseases. Biochim Biophys Acta 1862:114–129

Hsu C-D, Meaddough E, Basherra H, Harirah H, Lu L-C (2000) Increased apoptosis in human amnion is associated with labor at term. Am J Reprod Immunol 43:255–258

Ikebuchi Y, Masumoto N, Tasaka K, Kasahara K, Miyake A, Tanizawa O (1991) Superoxide anion increases intracellular pH, intracellular calcium, and arachidonate release in human amnion cells. J Biol Chem 266:13233–13237

Islam MN, Das SR, Emin MT, Wei M, Sun L, Rowlands DJ, Quadri SK, Bhattacharya S (2013) Mitochondrial transfer from bone marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nat Med 18:759–765

Iwasaki R, Matsubara S, Takizawa T, Takayama T, Yashiro T, Suzuki M (2003) Human amniotic epithelial cells are morphologically homogeneous: enzyme histochemical, tracer, and freeze-substitution fixation study. Eur J Histochem 47:223–232

Kovac S, Angelova PR, Holmström KM, Zhang Y, Dinkova-Kostova AT, Abramov AY (2015) Nrf2 regulates ROS production by mitochondria and NADPH oxidase. Biochim Biophys Acta, Gen Subj 1850:794–801

Kröller-Schön S, Steven S, Kossmann S, Scholz A, Daub S, Oelze M, Xia N, Hausding M, Mikhed Y, Zinssius E, Mader M, Stamm P, Treiber N, Scharffetter-Kochanek K, Li H, Schulz E, Wenzel P, Münzel T, Daiber A (2014) Molecular mechanisms of the crosstalk between mitochondria and NADPH oxidase through reactive oxygen species-studies in white blood cells and in animal models. Antioxid Redox Signal 20:247–266

Kumagai K, Otsuki Y, Ito Y, Shibata MA, Abe H, Ueki M (2001) Apoptosis in the normal human amnion at term, independent of Bcl-2 regulation and onset of labour. Mol Hum Reprod 7:681–689

Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4:181–189

Lee W, Johnson J, Gough DJ, Donoghue J, Cagnone GLM, Vaghjiani V, Brown KA, Johns TG, St. John JC (2015) Mitochondrial DNA copy number is regulated by DNA methylation and demethylation of POLGA in stem and cancer cells and their differentiated progeny. Cell Death Dis 6:e1664–e1611

Li C, Zhou H-M (2011) The role of manganese superoxide dismutase in inflammation defense. Enzym Res 2011:1–6

Lindström TM, Bennett PR (2005) 15-Deoxy-δ12,14-prostaglandin J2 inhibits interleukin-1β-induced nuclear factor-κB in human amnion and myometrial cells: mechanisms and implications. J Clin Endocrinol Metab 90:3534–3543

Liu W, Ruiz-Velasco A, Wang S, Khan S, Zi M, Jungmann A, Dolores Camacho-Muñoz M, Guo J, Du G, Xie L, Oceandy D, Nicolaou A, Galli G, Müller OJ, Cartwright EJ, Ji Y, Wang X (2017) Metabolic stress-induced cardiomyopathy is caused by mitochondrial dysfunction due to attenuated Erk5 signaling. Nat Commun 8:1–15

Masuyama M, Iida R, Takatsuka H, Yasuda T, Matsuki T (2005) Quantitative change in mitochondrial DNA content in various mouse tissues during aging. Biochim Biophys Acta, Gen Subj 1723:302–308

Matsubara S, Iwasaki R (2004) Golgi apparatus and mitochondria of human amniotic epithelial cells. Acta Histochem Cytochem 37:407–409

Menon R, Lombardi SJ, Fortunato SJ (2002) TNF-α promotes caspase activation and apoptosis in human fetal membranes. J Assist Reprod Genet 19:4–7

Mohammad G, Kowluru RA (2010) Matrix metalloproteinase-2 in the development of diabetic retinopathy and mitochondrial dysfunction. Lab Investig 90:1365–1372

Oh J, Lee YD, Wagers AJ (2014) Stem cell aging: mechanisms, regulators and therapeutic opportunities. Nat Med 20:870–880

Okamoto K, Kondo-Okamoto N (2012) Mitochondria and autophagy: critical interplay between the two homeostats. Biochim Biophys Acta 1820:595–600

Polettini J, Silva MG, Kacerovsky M, Syed TA, Saade G, Menon R (2014) Expression profiles of fetal membrane nicotinamide adenine dinucleotide phosphate oxidases (NOX) 2 and 3 differentiates spontaneous preterm birth and pPROM pathophysiologies. Placenta 35:188–194

Redza-Dutordoir M, Averill-Bates DA (2016) Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta 1863:2977–2992

Rizzuto R, De Stefani D, Raffaello A, Mammucari C (2012) Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol 13:566–578

Robin ED, Wong R (1998) Mitochondrial-DNA molecules and virtual number of mitochondria per cell in mammalian-cells. J Cell Physiol 136:507–513

Sabella N (1913) Use of fetal membranes in skin grafting. Med Rec NY 83:478–480

Sagol S, Sagol O, Ozkal S, Asena U (2002) Role of apoptosis, bcl-2 and bax protein expression in premature rupture of fetal membranes. J Reprod Med 47:809–815

Scherz-Shouval R, Elazar Z (2007) ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 17:422–427

Seo AY, Joseph A-MM, Dutta D, Hwang JC, Aris JP, Leeuwenburgh C (2010) New insights into the role of mitochondria in aging: mitochondrial dynamics and more. J Cell Sci 123:2533–2542

Shen ZY, Li EM, Lu SQ, Shen J, Cai YM, Wu YE, Zheng RM, Tan LJ, Xu LY (2008a) Autophagic and apoptotic cell death in amniotic epithelial cells. Placenta 29:956–961

Shen ZY, Xu LY, Li EM, Zhuang BR, Lu XF, Shen J, Wu XY, Li QS, Lin YJ, Chen YW, Tan LJ (2008b) Autophagy and endocytosis in the amnion. J Struct Biol 162:197–204

St. John JC (2016) Mitochondrial DNA copy number and replication in reprogramming and differentiation. Semin Cell Dev Biol 52:93–101

Stern M (1912) The grafting of preserved amniotic membrane to burned and ulcerated surfaces, substituting skin grafts. J Am Med Assoc LX:973–974

Telfer JF, Thomson AJ, Cameron IT, Greer IA, Norman JE (1997) Expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during normal human pregnancy and parturition. Hum Reprod 12:2306–2312

Than NG, Romero R, Tarca AL, Draghici S, Erez O, Chaiworapongsa T, Kim YM, Kim SK, Vaisbuch E, Tromp G (2009) Mitochondrial MnSOD mRNA expression in human chorioamniotic membranes and its association with labor, inflammation and infection. J Matern Neonatal Med 22:134.e1–134.e8

Thomas CE (1965) The ultrastructure of humon amnion epithelium. J Ultrastruct Res 13:65–84

Trinei M, Berniakovich I, Pelicci PG, Giorgio M (2006) Mitochondrial DNA copy number is regulated by cellular proliferation: a role for Ras and p66Shc. Biochim Biophys Acta Bioenerg 1757:624–630

Vaghjiani V, Cain JE, Lee W, Vaithilingam V, Tuch BE, St John JC (2017) Modulation of mitochondrial DNA copy number to induce hepatocytic differentiation of human amniotic epithelial cells. Stem Cells Dev 26:1505–1519

Wang W, Liu C, Sun K (2016) Induction of amnion epithelial apoptosis by cortisol via VtPA/plasmin system. Endocrinology 157:4487–4498

Weidinger A, Kozlov AV (2015) Biological activities of reactive oxygen and nitrogen species: oxidative stress versus signal transduction. Biomolecules 5:472–484

Weser H, Kaufmann P (1978) Lichtmikroskopische und histochemische Untersuchungen an der Chorionplatte der reiden menschlichen Placenta. Arch Gynakol 225:15–30

Youle RJ, van der Bliek AM (2012) Mitochondrial fission, fusion, and stress. Science 337:1062–1065

Molecular Crowding – (in Cell Culture)

Cedric Badowski, Aneesa Iskander, Diana Gaspar, Dimitrios I. Zeugolis, and Michael Raghunath

Contents

Abstract

Macromolecular crowding (MMC) is an intrinsic and ubiquitous feature in biological cells. We find MMC in the first bacterial cell and see it culminating in the intricate extracellular matrix (ECM) that evolved in multicellular organisms. Research work on MMC started with the observation that biological cellular

C. Badowski
Institute of Medical Biology, A*STAR, Singapore, Singapore
e-mail: cedricbadowski@gmail.com

A. Iskander · M. Raghunath (✉)
Institute for Chemistry and Biotechnology (ICBT), Department Life Sciences and Facility Management, ZHAW Zurich University of Applied Sciences, Wädenswil, Switzerland
e-mail: e0011348@u.nus.edu; ragh@zhaw.ch

D. Gaspar · D. I. Zeugolis
Regenerative, Modular and Developmental Engineering Laboratory (REMODEL) and Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM), Biomedical Sciences Building, National University of Ireland Galway, Galway, Ireland
e-mail: DIANA.GASPAR@nuigalway.ie; Dimitrios.Zeugolis@nuigalway.ie

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_50

systems are crammed with macromolecules. The interior of cells is teeming with enzymes, transport systems, and nucleotide assemblies. In addition, eukaryotic cells possess a three-layered cytoskeleton adding confinement to an already packed cytoplasm. Likewise, the extracellular space of multicellular organisms comprises an ECM consisting of fibrillar proteins, such as collagen or elastin, surrounded by an amorphous gel-like ground substance glycoproteins and proteoglycans and their hydration shells. Together, they provide mechanical resilience to the tissues of vertebrates while forming a crowded and structural microenvironment that in turn creates confinement for other macromolecules. Surprisingly, most biochemical and cell culture experiments are still done in non-crowded, highly aqueous solutions. Here, we shall discuss the shortcomings of contemporary cell culture and emphasize the benefits of applying MMC to cell culture models of tissues. MMC can be achieved by adding water-soluble macromolecules to the culture medium. Not only is this technically feasible, it also moves in vitro biology toward a higher physiological level, allowing the design of more meaningful cell-based assays and enabling tissue engineering of matured and physiologically relevant tissue-like assemblies.

1 Introduction

1.1 Shortcomings of Contemporary Cell Culture

Cell culture is a system that seeks to multiply cells extracted from living tissues in order to study them or to build new cell communities in vitro. So, first and foremost, the extracted cells need to stay alive. Cells come from a tissue context comprising neighboring cells and their microenvironment, the extracellular matrix (ECM). Cells are removed abruptly out of their optimal and complex tissue context (Cigognini et al. 2013) and are seeded usually on onto polystyrene, glass, or other surfaces. Robbed of their niche, cells must settle on a foreign surface, where they are expected to adhere, spread, and then divide by mitosis. At this point, it might be important to note that animal cells adhere to the proteins attached to the plastic dishes rather than the plastic itself. Indeed, plastic surfaces are treated to achieve fast binding of adhesive proteins, usually from serum, that mediate the actual cell adherence (Ramsey et al. 1984). Typical mediator proteins from serum are fibronectin and vitronectin, as they engage integrin receptors that all animal cells express on their surface (Hayman et al. 1985). Fibronectin is both an ECM and a serum protein. Serum fibronectin is a circulating biosynthetic product of the liver of the serum donor, while cellular fibronectin is made by the adhering cells (the vast majority of anchorage-dependent cells, if not all, are capable of producing one form of fibronectin or another) (Stenman and Vaheri 1978; Owens and Cimino 1982). As cells adhere to the serum proteins, they will add their own synthesized fibronectin to this. For example, human cells grown in the presence of fetal bovine serum will form a hybrid matrix of bovine and human fibronectin. Thus, the cells coat foreign surfaces with their own adhesive matrix proteins to facilitate their own anchorage. Obviously,

in vitro animal culture is a highly artificial system designed for optimal multiplication of cells, convenience, visual inspection, and optical studies of cell cultures. Getting cells to survive outside a tissue context is rather challenging, and decades of research and development have gone into the formulations of culture media that are commercially available to date. However, convenience issues seem to be getting in the way of redesigning cell culture despite scientific evidence that current cell culture systems are deficient on several levels. We will review these shortcomings only briefly (for more detailed reviews, please see Chen et al. 2011; Park et al. 2015), but it is important to be aware of them. Some of these shortcomings are not easily remedied, while others can be, as we will demonstrate in this chapter.

For a start, let us consider the cell attachment surface. Current surfaces are made of glass (still the best for optical imaging and short-term culture) (Mather and Roberts 1998), polystyrene (Curtis et al. 1983), and polycyclic olefins (Niles and Coassin 2008). These surfaces offer no intrinsic purchase for anchorage-dependent cells, other than perhaps strong electrostatic interactions with the cell membrane. Most of the 213 cell types of the human body are anchorage-dependent and would not thrive in suspension, unless they are transformed or cancer cells (Benecke et al. 1978). Therefore, surfaces are treated to make them negatively charged and hydrophilic so that polar regions of the proteins can stick to them and, in turn, mediate cell attachment via presentation of peptide sequences to cell receptors. Such well-described sequences are the RGD motifs that engage integrins (Ruoslahti 1996). Without the presence of those "sticky" proteins, cells will often not attach to plastic, as the experience of seeding cells in the absence of serum teaches us (Barnes and Sato 1980). Classical adhesion-mediating proteins are ECM proteins, which are secreted and immobilized outside of cells as supramolecular assemblies (Grinnell and Feld 1979). Cells have transmembrane receptors that bind to these ECM assemblies, and therefore, commercial ECM extracts or synthetic ECM-derived peptides contain integrin recognition sequences (e.g., nectins, laminins) (Aplin et al. 1998; Lutolf and Hubbell 2005). These peptides are used to coat cell culture plastic or come pre-coupled to plastic. In this regard, the current concept of "plastic adherence" for the isolation and characterization of mesenchymal stromal cells as a distinguishing criterion from hematopoietic progenitor cells from the bone marrow (Dominici et al. 2006), is quite intriguing. In reality, this is a selection of serum-protein-adherent cells or of cells particularly attracted to certain plastic surface charges. Emulating this on a more synthetic basis are peptides derived from the nectin family of proteins that are directly coupled to plasticware. One might wonder how different batches and qualities of serum or peptide coatings might decide over the very first selection of mesenchymal progenitor cells for subsequent expansion and later functionality and therapeutic application for tissue repair. Research in this direction might uncover interesting findings on the natural history of cultured mesenchymal stem cells.

This brings us to the missing microenvironment. Cells seeded onto culture vessels do not find an intricate ECM to settle in. No member of the family of collagens, laminins, nectins, microfibrillar proteins, and proteoglycans is present to welcome freshly seeded cells and help them anchor. Moreover, cell fate is highly dependent on

the surrounding microenvironment. Indeed, a particular set of ECM proteins could constitute a specific niche that would, for example, maintain stem cells, while a different ECM composition could favor differentiation into other cell types. The ECM is even more complex than it seems, since various matrix proteins can also bind different growth factors that promote cell growth and/or differentiation (Chen et al. 2007; Lin and Bissell 1993). To compensate for this lack of ECM diversity, artificial matrices meant to emulate extracellular matrix are continuously developed and published in biomaterials journals with the intention to supersede nature (Lutolf and Hubbell 2005; Kyburz and Anseth 2015). However, most coating materials commercially available for plastic ware are mostly ECM extracts and polymers of animal origin, either cell lines or tissue extracts. These products do not come cheap and, in many cases, would face regulatory hurdles for cell therapeutic intentions. Conceptually, these products reflect a disregard of the fact that the microenvironments the cells were harvested from was originally made by these very cells. *Obviously, freshly seeded cells are deprived of a fitting microenvironment to start with*; *however*, *we also need to understand that they are hobbled by the culture conditions in the first place*.

Culture media as such constitute another level of challenge in animal cell culture models. Although the development of culture medium spans several decades, it has remained essentially the same system: cells are fully immersed in a seawater equivalent supplemented with carbon sources (e.g., glucose, pyruvate, glutamine, and other amino acids), vitamins, varying micronutrients, and a carbon dioxide-based buffer system (unless HEPES is being used) (Bettger and McKeehan 1986; Eagle 1955). So-called high-glucose media are on the market, because they allegedly promote cell proliferation. It might be worthwhile to consider that high-glucose medium in essence reflects a diabetic condition, and therefore uncontrolled non-enzymatic glycation of cell cultures might occur in the long run and not reflect physiology (Brownlee 2001; Ahmed 2005).

The focus of this book chapter is that, except for the urothelium of the urinary bladder, the inner lining of blood vessels, and perhaps the synovia of joints, cells in vivo are not exposed to large amounts of water, unless in a pathological situation like an edema. Despite all the additives mentioned above, most culture media cannot sustain viability of cultured cells, at least not at the desired proliferation rates. Therefore, an enigmatic component is added in the range of 0.5–20% v/v – fetal bovine serum (FBS) (Van der Valk et al. 2010; Arigony et al. 2013; Honn et al. 1975). FBS contains an array of hormones and stimulatory factors (Zheng et al. 2006), along with the abovementioned adhesive components: fibronectin and vitronectin. The global supply of FBS is limited; it comes from different countries with considerable batch to batch variations, leading to different proliferation and functionalities of cells cultured in those respective serum batches (Gstraunthaler et al. 2013). While endothelial cells might be continuously exposed to serum (plasma) proteins in some way, most other cell types might never see serum components in their entire life span, unless under pathological circumstances, such as leaky vasculature during inflammation or a wound. Current discussions to remove fetal bovine serum from culture media pivot therefore on reproducibility, concerns

on transfer of xenogeneic pathogens and immunology, as well as issues of animal welfare related to the harvest of the biological product (Van der Valk et al. 2004). However, an often lackluster performance of cells in serum-free preparations in combination with high costs has prevented a deeper penetration of serum-free culture media at present in research laboratories.

Growing cells in the classical incubator is performed in the dark, at a humid atmosphere of 5% carbon dioxide, atmospheric oxygen (20%), and at 37 °C. Firstly, the inside of a dark incubator might not provide sufficient electromagnetic stimulation (light) for cell types harvested from the body surface (corneal epithelium, dermal and epidermal cells, such as keratinocytes and melanocytes). More importantly, oxygen levels in arterial blood range from 9.5% to 13% and drop, in actual tissues, to as low as 0.5% (Mohyeldin et al. 2010). Atmospheric CO_2 is 0.04% but reaches 4.6% and 6% in tissues (Hoffman et al. 1998; Bolevich et al. 2016). It follows that cells are routinely grown in hyperoxic and thus stressful conditions, possibly leading to faster aging of cell cultures or cell lines that genetically drift due to the incorporation of mutations associated with reactive oxygen species (ROS). This hyperoxic environment may also affect cell fate since numerous stress-related pathways are involved in cell differentiation, notably by activating stress-activated protein kinases such P38 and JNK kinases (Lee and Choi 2003). Temperature wise, 37 °C might be ever only reached in the liver bed. Elsewhere, temperatures are typically lower by 2–3 °C (Werner and Buse 1988; Birnie and Grayson 1952). Although shear forces will be present in bioreactors with flow and cell cultures on microcarriers in suspensions that are continuously stirred (Ratcliffe and Niklason 2002), the absence of mechanical stimulation in conventional cell culture must also be mentioned here. Reintroducing macromolecular crowdedness into culture media, by ways of adding macromolecules, could not only remedy the wateriness of the culture medium but also solve the issue of insufficient microenvironment formation.

2 The Long and the Short of Macromolecular Crowding

The first application of macromolecular crowding in cell culture goes back 30 years. Firstly polyethylene glycol (4 kDa, 5% w/v), polyvinylpyrrolidone (30–40 kDa, 6.5% w/v), and dextran T-40 (40 kDa, 5% w/v) (Bateman et al. 1986) and later dextran sulfate (500 kDa, 0.01% w/v) (Bateman and Golub 1990) were shown to increase the conversion of procollagen to collagen. The authors showed an increased deposition of collagen in the ECM by fibroblasts in order to highlight the activity of bone morphogenetic protein 1, then known as procollagen C proteinase (Bateman et al. 1986). Thus, a method had been described to increase collagen deposition in vitro. It was also used to study collagen deposition by fibroblasts from a patient with osteogenesis imperfecta (Cetta et al. 1993), and in a cell-free system, dextran sulfate or polyethylene glycol was shown to speed up the activity of the same enzyme purified from chick embryo tendons (Hojima et al. 1994). In these publications, the terms “excluded volume” and “macromolecular crowding” were used, respectively, but the mechanism leading to an increased procollagen cleavage and collagen

deposition remained unclear; a protein precipitation and aggregation step of some kind were discussed at that time. In the last 10 years, we and others have been able to gain more mechanistic insights into the effects of MMC in cell culture.

All considerations of macromolecular crowding start with the notion that the interior of a cell is crammed with macromolecules. In addition, eukaryotic cells contain a cytoskeletal meshwork that adds confinement to this macromolecular system. These considerations have been reflected in numerous review articles (Minton 2001; Ellis 2001). But when we take a closer look at the extracellular space in multicellular organisms, we find a comparable situation: the immediate vicinity of cells is also crammed with macromolecules, many of the immobilized, thereby adding confinement to the microenvironment. Therefore, any molecule secreted into the extracellular space will encounter a crowded environment in vivo.

Another important point to consider is that, in chemical reactions, reactants are modeled as point masses. This means that the actual sizes of the reaction partners are negligible and are therefore indeed neglected. In biochemical reactions, however, size does matter. In comparison with their respective substrate, enzymes can be huge, for example, glucose (180 Da) and glucose oxidase (160 kDa) (Woolridge et al. 1986), or are dwarfed by it like DNA polymerases working on a DNA strand, or exhibit similar masses such as bone morphogenetic protein 1 (88 kDa) (Lee et al. 1997) and procollagen (100 kDa) (Bornstein 1974). This means that the size of a macromolecular crowder might matter substantially with regard to the sizes of the molecules partaking in a biochemical reaction that are to be modulated by MMC.

3 Paradigm Shift: Make Your Own Matrix Scaffold In Vitro

The ECM of tissues is composed of fibrillar proteins and a gel-like ground substance made of dozens of glycoproteins and proteoglycans that are secreted by and immobilized around matrix-producing cells (Theocharis et al. 2016). It is important to distinguish between the biosynthesis of ECM molecules, their secretion, and their extracellular assembly into water-insoluble structures – the *deposited* ECM (Mouw et al. 2014). Clearly, secretion does not necessarily follow biosynthesis, and deposition does not necessarily follow secretion.

The biosynthesis of ECM molecules depends on signals that are received by the cell, such as growth factors or breakdown products of ECM. The secretion of a variety of collagens, however, is greatly aided by the presence of vitamin C (Murad et al. 1981). The extracellular assembly that follows successful secretion is a supramolecular aggregation of partners forming polymeric fibrils, or mesh-like structures. The basic polymeric structures are represented by fibronectin, collagens, laminins, and elastic microfibrils (Theocharis et al. 2016). Some collagens are forming fibrils, such as types I, II, and VII, while other collagens use these already formed fibrils as assembly points without forming fiber systems, such as type IX and XI collagens (Gelse et al. 2003). In analogy, elastin binds to fibrillin-containing microfibrils. Others collagens like collagens III and V mix to form heterotypic fibrils with collagen I and form the bulk of the connective tissue of the dermis (Birk et al.

1990; Fleischmajer et al. 1990). These biopolymeric assemblies represent molecular landing sites for a variety of ligands that in turn fasten growth factors to the ECM. Examples here are latent transforming growth factor beta-binding protein (LTBP) binding to fibronectin or elastic microfibrils and capturing latent TGFbeta and negatively charged proteoglycans associated with fibrillary structures that capture positively charged growth factors, like FGF and VEGF. Finally, enzymatic and nonenzymatic modifications of these supramolecular assemblies lead to the maturation of the built cell microenvironment. This includes later remodeling, involving breaking down structures while making way for new ones and changes in shape, structure, and composition of this ECM (Royce and Steinmann 2003).

Earlier work suggests that macromolecular crowding can aid in enzymatic reactions by stabilizing complexes of enzyme and substrate, thus increasing enzymatic activities (Zhou et al. 2008). Interestingly, additional effects are seen through the deposition, stabilization, and remodeling of ECM and are dependent on a variety of enzymes (Fig. 1). To select a few, the intracellular posttranslational modification of collagen that is crucial to its thermo-stabilization and therefore successful release into the extracellular space is catalyzed by prolyl hydroxylase (Notbohm et al. 1992). The extracellular proteolytic conversion of procollagen to collagen is controlled by bone morphogenetic protein 1 (also known as procollagen C proteinase) activity (Fig. 1). The extracellular covalent crosslinking of collagens (and elastin) is effected by the copper-dependent enzyme lysyl oxidase and the calcium-dependent transglutaminase 2 (Wang and Griffin 2012). Remodeling, removing, and reshuffling of the ECM are performed by matrix metalloproteinases and other proteolytic enzymes (Bonnans et al. 2014). The activation of these ECM-modifying enzymes sometimes requires their own cleavage by proteolytic enzymes. Therefore, multiple potential targets for MMC with regard to enzyme-mediated modifications in the extracellular space arise (for comprehensive overview, see Royce and Steinmann 2003).

The first ECM-relevant observation with macromolecular crowding focused on the procollagen-converting enzyme bone morphogenetic protein 1/procollagen C proteinase. Macromolecular crowding induces faster conversion of procollagen to collagen, leading to stronger presence of cleaved collagen in the pericellular matrix and a correspondingly weaker presence of uncleaved procollagen in the culture medium (Bateman et al. 1986; Bateman and Golub 1990). In a cell-free system, the proteolytic activity of procollagen C proteinase on procollagen was found to be accelerated under MMC (Hojima et al. 1994). The addition of crowders to the culture medium was also shown to increase the conversion of procollagen to collagen in cell culture; immunochemical analysis demonstrated a substantial increase of the clipped-off collagen I C-propeptide trimer under crowding (Chen et al. 2011). Moreover, the authors demonstrated that procollagen C proteinase enhancer (PCPE), an allosteric regulator of procollagen C proteinase, was also proteolytically converted into an active enhancer of procollagen C proteinase activity and a smaller fragment that is speculated to have anti-collagenolytic properties, similar to tissue inhibitors of metalloproteinases (TIMP) (Mott et al. 2000). Interestingly, PCPE also activates the crosslinking enzyme lysyl oxidase (Trackman 2005). Our work demonstrated that MMC accelerated more than one

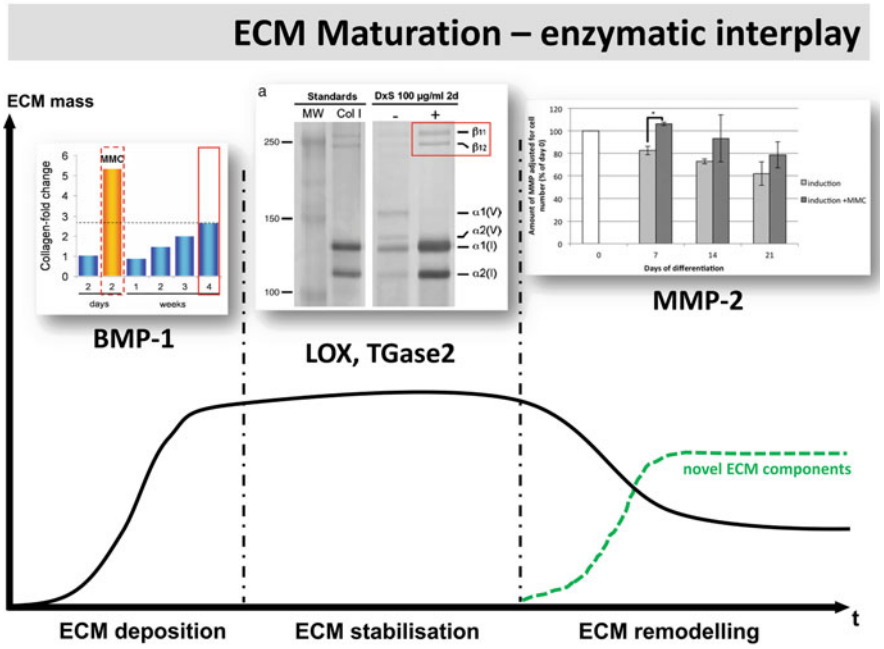


Fig. 1 Enzymatic key activities in the formation and maturation of ECM that are targets of MMC. Left: extracellular collagen deposition is boosted by accelerating bone morphogenetic protein 1 (*BMP1*), also known as procollagen C proteinase. The enzymatic activity shortens procollagen to collagen triple helices by removing globular C-termini. This trimming process now allows the assembly of collagen molecules to form insoluble fiber systems. This enzymatic process is rate-limiting for collagen deposition and slow in standard culture (blue columns). Therefore, the acceleration of this step via MMC (orange column) represents a substantial advantage in cellular microenvironment formation. (Reprinted from Lareu et al. 2007b, with permission from the publisher, Elsevier Center: Enzymes play a crucial role in the stabilization of deposited ECM). Shown here are high molecular weight double bands (red box) representing collagen alpha chains that have been covalently crosslinked by lysyl oxidase (*LOX*) activity. (Reprinted from Chen et al. 2011, with permission from Elsevier). Right: the remodeling of ECM involves the proteolytic removal of already deposited matrix components and often is accompanied by the deposition of new matrix. Therefore, not only the quantity but also the composition of the ECM will change as some ECM components will recede while others emerge. Here, matrix metalloproteinase 2 (*MMP2*) has been demonstrated via zymography to be strongly associated with matrix during adipogenesis under MMC. (Reprinted from Ang et al. 2014 with permission from Mary Ann Liebert Inc.)

converting enzyme in a cascade process. We also speculated that the binding of PCPE to procollagen C proteinase would be faster and tighter under crowding as well, which would have a tremendous effect, as PCPE is known to increase the catalytic activity of procollagen C proteinase by one order of magnitude (Lareu et al. 2007a) (Fig. 1).

Once procollagen is cleaved to collagen, the collagen molecules assemble as trimers in an orderly fashion, first forming nuclei and then extending and elongating into microfibrils that grow in length and girth. In parallel, crosslinking enzymes

stabilize these meshworks and assist to immobilize them and protect them against proteolytic attack (Bornstein 1974). Lysyl oxidase crosslinks have especially been described and investigated extensively in various connective tissues ranging from tendon, skin, and bone to cornea and blood vessels (Kielty and Grant 2003). Transglutaminase 2 also plays a role in covalently crosslinking collagen assemblies (Wang and Griffin 2012; Raghunath et al. 1999; Raghunath et al. 1996; Zeugolis et al. 2010) as well as affixing ligands to ECM assemblies such as latent transforming growth factor binding proteins (Raghunath et al. 1998; Taipale et al. 1996) (Fig. 1).

However, ECM also requires remodeling, when tissue changes arise. Obviously, growth of a tissue and repair processes after injury require the addition and the rearrangement of the ECM. One classic example is the cascade of events that occurs after the fracture of a bone (Alford and Hankenson 2006; Damsky 1999). Firstly, the adjacent ends of the fractured bone will be partially resorbed via decalcification and removal of collagen fibers, and then a collagenous soft tissue occurs between the borders of the fracture, the callus, which then finally gets fully calcified. Damaged and denatured ECM will be proteolytically removed, and new ECM needs to be deposited and welded to pre-existing structures. In this case, the connective tissue is replaced with a similar connective tissue. Cell differentiation processes also require remodeling not only in terms of shape but also in terms of composition. The formation of adipose tissue, for example, requires a drastic change of progenitor cells from spindle shaped to spherical as demanded by the intracellular accumulation of lipid droplets (Nakajima et al. 1998; Gregoire et al. 1998). In consequence, the ECM, having to accommodate different cell shapes, will switch from a collagen I- and fibronectin-rich ECM to an ECM dominated by collagen IV, invoking the reduction of fibronectin. This sequence of events in adipogenesis involving proteolytic remodeling of the ECM was first proposed in culture in murine pre-adipocyte 3T3-L1 cells (Lilla et al. 2002; Selvarajan et al. 2001) and was confirmed later in human cells by differentiating human bone-derived mesenchymal stromal cells under MMC in vitro (Ang et al. 2014). Here, proteolytic enzymes that are specific to ECM components and assembly thereof come into play. We have shown that MMP-2 is more closely associated with the ECM under crowding conditions and that MMP activity can be accelerated under crowding conditions in a cell-free system (Ang et al. 2014) (Fig. 1).

4 Not to Be Overlooked: Collagens and Ascorbic Acid

We have shown that MMC greatly accelerates enzymatic processes that facilitate collagen matrix formation and deposition, which are normally tardy in standard cell culture. However, it cannot be stressed enough that any of these enzymes can only work with ECM components that are available in the pericellular space. This is where ascorbic acid (Vitamin C) comes in, as it is responsible for the efficient secretion of procollagen (Graham et al. 1995). Ascorbic acid is a crucial cofactor for the enzymes prolyl hydroxylase and lysyl hydroxylase, both major posttranslational modifiers of procollagen in the endoplasmic reticulum. While hydroxylation

of the frequent lysyl residues in collagen plays a role later for collagen crosslinking, hydroxylation of the prolyl residues in procollagen immediately determines its thermal stability and thus its folding state (Yamauchi and Sricholpech 2012). The term procollagen refers to a triple helix composed of three procollagen alpha chains. In the endoplasmic reticulum, three such procollagen alpha chains form a procollagen triple helix. The assembly of the three alpha chains in a triple helix starts at the C-terminus, in a zipper-like fashion, toward the N-terminus. This process takes roughly a quarter of an hour for one triple helix (Raghunath et al. 1994). During folding, several enzymes posttranslationally modify the nascent triple helix. Because a folded triple helix is not easily accessible to these enzymes, they are "surfing" along the procollagen alpha chains ahead of the folding front to fulfill their tasks. All biochemical processes are in equilibrium. This means for collagen triple helices that they are continuously folding and unfolding ("breathing"). As only a completely folded procollagen can leave the endoplasmic reticulum, the equilibrium at body temperature must be shifted to the fully folded state. Here, prolyl hydroxylation comes into play by generating hydroxyprolines that help locking the triple helix in the folded state. In the absence of ascorbic acid, collagen biosynthesis can still occur but under-hydroxylated procollagen populations will be retained within the cell because they are not properly folded. It follows that ascorbic acid is crucial for an efficient collagen secretion (Barnes 1975). Vitamin C cannot be synthesized by humans, making it an essential vitamin that must be ingested. Nutritional Vitamin C deficiency, known as scurvy, leads to a paucity of extracellular collagen. This has dramatic effects on wound healing, blood vessel integrity, and periodontal health, all pointing to severe impairment of ECM maintenance (Peterkofsky 1991). It was estimated that British troops "even in World War I had more scurvy than gunshot casualties" (Kiple 2002) and that prior to the twentieth century, more soldiers and sailors deployed in warfare succumbed to scurvy than died in battle or accidents (McDowell 2013).

While scurvy might now be less frequent in modern societies, it is often involuntarily reproduced in ascorbic acid-free cell cultures where we find cells intracellularly retaining their procollagen, while the little that is secreted extracellularly hardly forms a pericellular matrix. The sensitivity to under-hydroxylation appears to vary between collagen and cell types and may depend on the percentage of hydroxylated prolyl residues per procollagen alpha chain and the specific thermostability pattern of the collagen type in question. For collagen I, a minimum of 120 hydroxylated prolyl residues is required to achieve thermostability (Royce and Steinmann 2003). While collagen I and VII may be the most sensitive to vitamin C deficiency in vitro, observations with collagen IV deposition suggest that it might be less vulnerable, but no systematic studies exist here. While MMC facilitates ECM deposition, it also depends on full collagen secretion to unlock its greatest benefits in building a "homemade" ECM microenvironment for cells in culture. Other ECM components are not dependent on the role of ascorbic acid such as fibronectin or elastic microfibrillar components. Therefore, an ECM built in the absence of vitamin C would preferably contain non-collagenous components and therefore present with a skewed stoichiometry. This, however, also would offer an opportunity of modulating ECM compositions using MMC.

5 Macromolecular Crowding in Macromolecular Assemblies

Collagen gels can be generated in vitro exploiting the intrinsic self-assembly process of collagen triple helices. Solutions of collagen containing pretrimmed procollagen can be made or bought. Collagen molecules are prevented from fibril formation by keeping them at very low pH, for example, in 1 M hydrochloric acid or 0.5 M acetic acid. When this solution is brought to neutral pH, the collagen assembly process begins rapidly, resulting in the formation of a gel made of collagen fibrils in a haphazard arrangement. This way of making collagen hydrogels is very popular, and collagen gels are used for a variety of assays and culture systems (Collin et al. 2011). The process of gel formation, which is essentially a process of fibrillogenesis, can be followed via turbidimetry. Light is shone through a cuvette containing the collagen solution; when fibrillogenesis occurs, the light is scattered because of the forming fibrils that render the solution turbid. Collagen gel formation is described in turbidimetry by two phases. Firstly, multiple foci form, consisting of a few collagen triple helices forming nuclei. This is called the lag phase, and while there is no apparent signal in turbidimetry, the formation of numerous nuclei is happening. Then, the solution starts getting turbid rapidly because of the nuclei that are now elongated into linear structures forming proper fibrils. This is the growth phase. The absorption curve shows a steep slope during this phase and a plateau when all collagen triple helices are spent in the process of fibrillogenesis (Dewavrin et al. 2015) (Fig. 2). When we first experimented with negatively charged macromolecular crowders (e.g., dextran sulfate), gel formation would occur so rapidly that turbidimetry was impossible to do (unpublished). We then studied the effects of single neutrally charged crowders to capture changes of fibril formation quantitatively (Dewavrin et al. 2015, 2014). MMC appeared to shorten the lag phase of collagen fibrillogenesis by creating a greater number of elongation nuclei in a shorter time window while the rate and extent of elongation (visible as the slope) remained unchanged. However, a plateau was reached faster, which suggested that crowding leads to the formation of more but thinner collagen fibrils (Fig. 2). These cell-free in vitro data on collagen fibrillogenesis were later confirmed in monolayer cell culture systems with the basement membrane collagen IV in the adipogenic differentiation of human bone marrow-derived stromal cells and progenitors from the stromal vascular fraction of human subcutaneous fat. Here, brown fat cells generated under macromolecular crowding were embedded in a collagen IV cocoon consisting of thin fibrils, whereas non-crowded cultures showed coarse and thick collagen bundles framing but not enveloping cells (Lee et al. 2016) (Fig. 3).

The assembly of collagen has now been extensively studied under macromolecular crowding in cell-free systems and in monolayer cultures of collagen-producing cells. The primordial ECM molecule, however, is fibronectin. It is produced and laid down first by cells (and or grabbed from serum as discussed above) and serves as a template for collagen deposition (Kadler et al. 1996). While we have not systematically studied fibronectin ECM formation in vitro, we have shown that macromolecular crowding does promote fibronectin deposition in vitro in mesenchymal stem cells seeded in the absence of serum (Chen et al. 2011). However, more work needs to be done to study self-assembly of fibronectin under MMC.

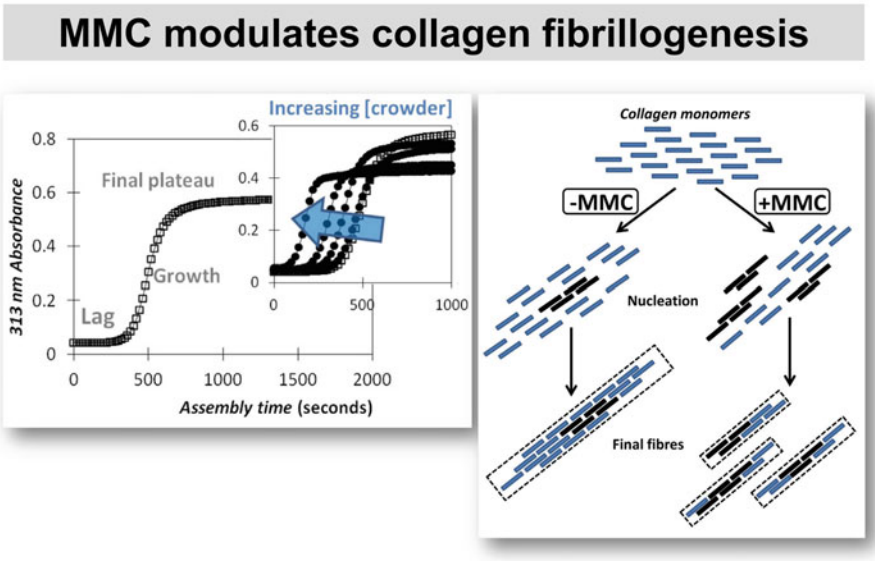


Fig. 2 Effects of mono-crowding using 400 kDa on the kinetics of collagen I gel formation. The starting material is an acidic solution containing single collagen triple helices. Upon neutralization of this solution, the collagen fiber assembly starts and a collagen gel forms. Left panel: phases of collagen assembly as assessed by turbidimetry at 313 nm: No apparent signal is obtained before and during nucleation (lag time). The phase of elongation of nuclei (fiber growth) becomes visible with an increased absorption and a final A313 plateau, denoting the conclusion of collagen assembly. Right panel: the generation of an increased number of nuclei under MMC leads to the formation of more but finer collagen fibers. (Reprinted from Dewavrin et al. 2014, with permission from Elsevier)

We have shown that MMC promotes ECM formation at least on two levels: on the enzymatic level and on the supramolecular assembly level (see Figs. 1 and 2). Thus, the logic of applying macromolecular crowding to empower cells to build their own microenvironment appears quite compelling. While adding macromolecular crowders to culture medium is straightforward, the relevant biophysics, however, are highly complex and have been subject to in vitro and extensive in silico considerations. These considerations can be found in more detail in a number of pertinent publications, some of which are cited throughout this text. One of these considerations is that in any given volume, macromolecular crowders occupy space. If we assume that all molecules in such a given volume are spherical and that we know their number (molarity) and their size (hydrodynamic radius, R_H), we can then calculate the volume they occupy and set this in relation to the given volume they are in. Thus, the fractional volume occupancy (FVO) with the mathematical symbol Ψ can be determined, allowing a first approximation of crowdedness in a given system. Biological systems show a Ψ in the range of 5–32% and are expressed as volume/volume (Fig. 4) (Chen et al. 2011).

For all intents and purposes, we shall equate this FVO with the excluded volume, which means that the crowder-occupied space excludes all other molecules from that space. This, of course, only applies for truly globular molecules and is a generalization

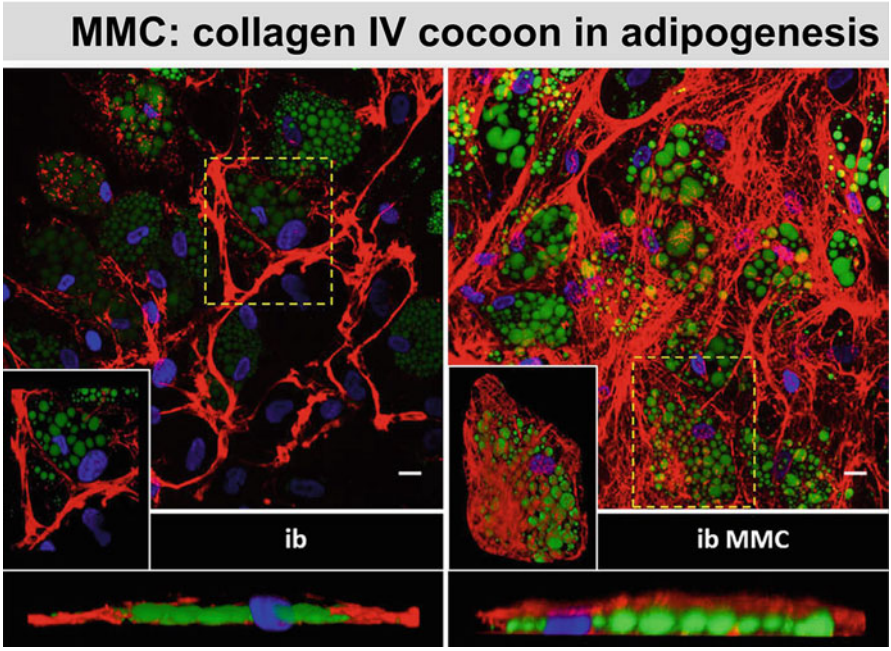


Fig. 3 MMC enhances formation of Col IV basement membrane architecture during adipogenic differentiation. Human bone marrow-derived stem cells were differentiated into brown adipocytes under standard culture conditions (left panels) and under mixed macromolecular crowding using Ficoll (right panels). Z-stack images were obtained for each condition through confocal microscopy. The Z-project images show nuclei (blue), Col IV (red), and lipid droplets (green). The inserts and cross-sections are the reconstructed 3D images of the selected cells boxed in yellow to show the pericellular distribution of Col IV. Scale bar: 20 μm. Bottom images represent cross-sections of the region of interest. The cross-section reveals that only under MMC single adipocytes are fully wrapped in a collagen IV cocoon. This situation would be normally seen only in cells seeded into hydrogels. Thus, MMC creates an ultra-flat 3D setting with regard to ECM distribution in a monolayer. (Reprinted from Lee et al. 2016, under Creative Commons CC-BY-4.0 license. Available from: https://doi.org/10.1038/srep21173)

that will not characterize all macromolecules well. For example, a collagen triple helix is a rod-like trimer with a diameter of 1.5 nm and a length of 280 nm, as aspherical as a molecule can get (Shoulders and Raines 2009; Lodish et al. 2000). However, assumptions must be made to get to grips with any system, and this approach has a time-honored tradition in engineering. Basically, the power of macromolecular crowding resides in its ability to confine reactants to less space, thereby leading to a virtual increase in concentration of reactants. However, macromolecular crowding does much more than just making reaction volumes smaller or reducing the volume of culture media. We have seen a moderate increase of collagen deposition in greatly reduced culture medium (200 μl versus 500 μl) in 24-well plates (Chen et al. 2011). While we created a concentration effect in these cell cultures, we put them in danger of drying out or running out of nutrients fast. Moreover, just reducing the culture medium volume

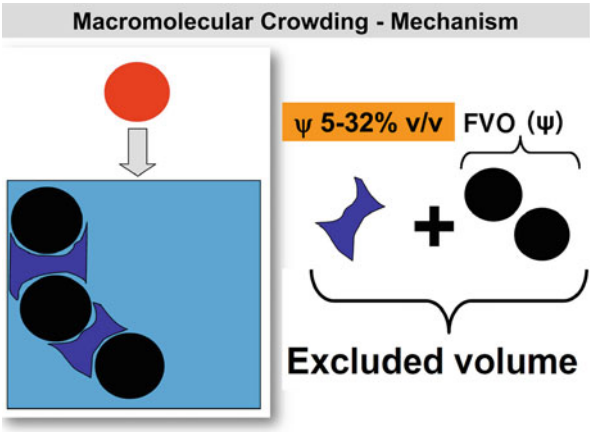


Fig. 4 A simplified representation of the generation of the excluded volume effects through the presence of macromolecular crowders in culture medium. Here, a test molecule (red) is about to enter a given volume (blue box). The given volume already contains three macromolecules (crowders, black). If we assume that these crowders occupy 30% of this volume, their fractional volume occupancy (*FVO*, Ψ) would be given as 30% (v/v). The FVO can be calculated when the volume is known, along with the number of crowders (molarity) and their volume. The volume can be calculated assuming the crowders to be spherical. In this case, the hydrodynamic radius will be used. The additional unavailable volume is a challenge to calculate, as several factors such as electrostatic repulsion and hydration shell need to be considered. Water molecules and Brownian motion are not considered in this model. (Reprinted from Chen et al. 2011, with permission from Elsevier)

will not change the relationship between total volume and available volume. In contrast, with a crowded standard culture medium volume (500 µl), we saw much stronger deposition effects because the thermodynamic activity is much larger in a crowded culture medium, which has simply to do with the substantially reduced available volume versus a total given volume (Fig. 5).

6 The Power of Charge and More: Mixed Macromolecular Crowding

Macromolecular crowding can be created using a single size species macromolecule, and most model systems work with one crowder; however, the combination of two or more crowders is more powerful than a system with only one crowder. A selection of commonly used crowders can be found in Table 1. We shall use the term "mono-crowding" for usage of only one size of one particular macromolecular crowder and "mixed macromolecular crowding" for usage of two or more crowders with different molecular weights. The molecular weight, Mw, of a given crowding molecule would cause steric hindrance by occupying space. At physiological pH, most proteins are negatively charged, depending on their amino acid composition and their respective

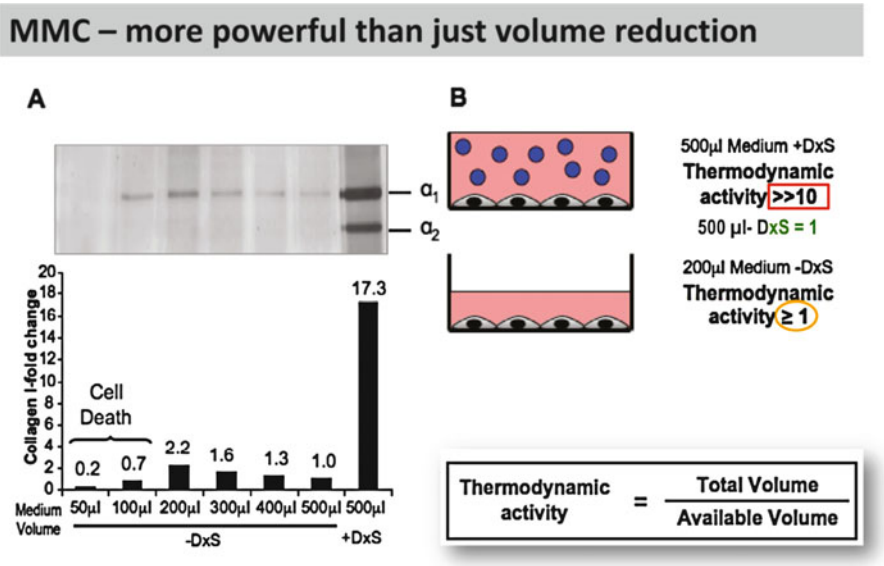


Fig. 5 Medium volume reduction effects are negligible compared with macromolecular crowding of the culture medium. (**a**) Fibroblast cultures were grown under non-crowded conditions in decreasing medium volumes. Their collagen matrix deposition was compared with cells in the highest volume under MMC with dextran sulfate (*DxS*). A 2.2-fold increase of collagen deposition was indeed seen in 200 µl but was clearly outperformed in DxS-crowded fibroblasts (17-fold increase) in 500 µl. In 100 µl and 50 µl, respectively, wells dried out, resulting in cell death. (**b**) The ratio between total volume and available volume will always stay 1 under non-crowded conditions, regardless of the actual volume in the cell culture as both volumina do not change in their relationship to each other. For 500 µl standard culture, the ratio is 1 (green text), as well as in 200 µl. However, it could be argued that in a smaller volume, cellular secretion of macromolecules might introduce some form of MMC, as well, so reducing the available volume slightly. However, thermodynamic activities in 500 µl of DxS-crowded medium increase dramatically, as the available volume is substantially reduced under MMC. (Copyright 2010 Peng Y, Raghunath M. Published in (Peng and Raghunath 2010) and reproduced with permission under CC-BY-NC-SA 3.0 license. Available from: https://doi.org/10.5772/8573)

cumulative isoelectric points. Consequently, a negatively charged crowder would have an even greater volume-excluding effect due to electrostatic repulsion of negatively charged molecules by negatively charged crowders (Harve et al. 2006). In fact, we have demonstrated that the hydrodynamic radius (R_H) of negatively charged crowders is substantially larger in physiological salt solutions than a comparably sized neutrally charged crowder (Lareu et al. 2007b). The resulting larger volume-excluding effect that virtually increases reactant concentration could explain the extremely accelerated collagen deposition seen in presence of dextran sulfate 500 kDa (Lareu et al. 2007a, b). The size differences, as described by the hydrodynamic radius, also explained why we saw much weaker effects with mono-crowding with neutrally charged dextran or Ficoll molecules. Full collagen deposition under dextran sulfate was effectively enhanced in vitro within 48 h with fetal lung

Table 1 Commonly used macromolecular crowders for mono- and mixed crowding, cell types analyzed, and overview of relevant outcomes

Crowder	Cell types tested	Outcome	Reference
Dextran sulfate 10 kDa	WI-38 fibroblasts	Accelerated conversion of procollagen to collagen	Lareu et al. (2007a)
Dextran sulfate 500 kDa	WI-38, WS-1, corneal, dermal, and hypertrophic scar fibroblasts	Increased ECM deposition, accelerated C-propeptide cleavage, myofibroblastic differentiation of corneal fibroblasts	Bateman and Golub (1990), Lareu et al. (2007a, b), Chen et al. (2009), Satyam et al. (2014), and Kumar et al. (2015b)
Dextran T40	Dermal fibroblasts	Full processing of procollagen to collagen	Bateman et al. (1986) and Jukkola et al. (1991)
Dextran 670 kDa	WI-38 and hypertrophic scar fibroblasts	Increased ECM deposition	Lareu et al. (2007b)
Polystyrene sulfonate 200 kDa	WI-38 fibroblasts	Increased ECM deposition	Lareu et al. (2007a)
Polyethylene glycol 4 kDa	Dermal fibroblasts	Full processing of procollagen to collagen	Bateman et al. (1986)
Polyvinylpyrrolidone 40 kDa, 360 kDa	Bone marrow stem cells, dermal fibroblasts	Increased ECM deposition, increased cell proliferation (with 360 kDa)	Rashid et al. (2014)
Carrageenan	WI-18 and WS-1 fibroblasts, bone marrow stem cells	Increased ECM deposition modulated by crowder polydispersity	Satyam et al. (2016, 2014), and Cigognini et al. (2016)
Ficoll 70 kDa	WI-38 fibroblasts	No increase in ECM deposition	Lareu et al. (2007a)
Ficoll 400 kDa	WI-38 fibroblasts	No increase in ECM deposition. Modulation of fibrillogenesis kinetics of reconstituted kidney ECM	Lareu et al. (2007a) and Magno et al. (2017)
Cocktail of Ficoll 70 kDa and 400 kDa	Bone marrow stem cells, chondrocytes, WI-38, WS-1, and corneal fibroblasts	Increased collagen deposition, increased glycosaminoglycan deposition	Chen et al. (2009), Satyam et al. (2014), Rashid et al. (2014), Cigognini et al. (2016), Zeiger et al. (2012), Ang et al. (2013), and Kumar et al. (2015a)
Cocktail of dextran sulfate 10 kDa, Ficoll™ 70 kDa and Ficoll™ 400 kDa	Bone marrow stem cells	Increased deposition of ECM and growth factor retention	Prewitz et al. (2015)

fibroblast lines, as more collagen was detected within the matrix, as compared to cells cultured for several weeks without macromolecular crowding (Lareu et al. 2007b; Chen et al. 2009).

However, the deposited collagen (and fibronectin) matrix appeared granular; ultrastructural imaging revealed these granules to contain thin cross-striated collagen fibrils, but a contiguous collagen meshwork was not apparent under negative mono-crowding, (Lareu et al. 2007b). We also came to realize that not every cell line thrives under these crowding conditions. We suspected that dextran sulfate, and likewise other negatively charged crowders, might interfere with a variety of growth factors that have a positive net charge. We initially saw weaker results using single neutrally charged crowders (Lareu et al. 2007a) but were inspired by modeling work predicting that the combination of two differently sized crowders would generate a stronger volume occupancy/exclusion effect (Zhou 2008). We therefore combined the sucrose copolymers Ficoll 70 kDa with Ficoll 400 kDa and after a few titration rounds determined that mixed macromolecular crowding had indeed a strong, albeit slower than dextran sulfate, effect on collagen deposition (Lareu et al. 2007a; Chen et al. 2009). The current Ficoll mixture we have been using ever since has a theoretical FVO of 18% v/v, which we determined to be equivalent to the albumin fraction in serum (Chen et al. 2011b).

Using cell-free collagen assembly as a readout system, we could experimentally confirm previous predictions (Zhou 2008) that the combination of a minimum of two differently sized species of crowders indeed creates more excluded volume than theoretically expected and discussed the underlying theory (Fig. 6) (Dewavrin et al. 2015). We have used mixed macromolecular crowding successfully also in settings of enzymatic reactions, such as PCR (Lareu et al. 2007a) and in DNA hybridization experiments (Harve et al. 2010). The advantage of mixed macromolecular crowding using Ficoll is that two synthetic polymers of defined size give a controlled biphasic size distribution pattern. However, theoretical and experimental data would suggest that a third or fourth crowder in descending size might even close more spaces. Indeed we used triple crowding in PCR and hybridization experiments (Harve et al. 2010). Therefore, reproducible polydispersity would be an interesting approach, as it would represent a bandwidth of different sizes. This approach has been put into practice using carrageenan, a polydisperse macromolecule that has shown powerful ECM deposition in vitro (Satyam et al. 2014; Kumar et al. 2015a, b; Cigognini et al. 2016; Satyam et al. 2016; Kumar et al. 2018). Therefore, mixed macromolecular crowding should be attempted in vitro wherever possible (Fig. 6).

7 Cell Sheet Engineering and Macromolecular Crowding: A New Match

Cell sheet engineering is based on growing cell layers on a thermosensitive polymer at body temperature and then to lift the cells off, along with their deposited ECM, as one coherent layer when the culture vessel is placed in a

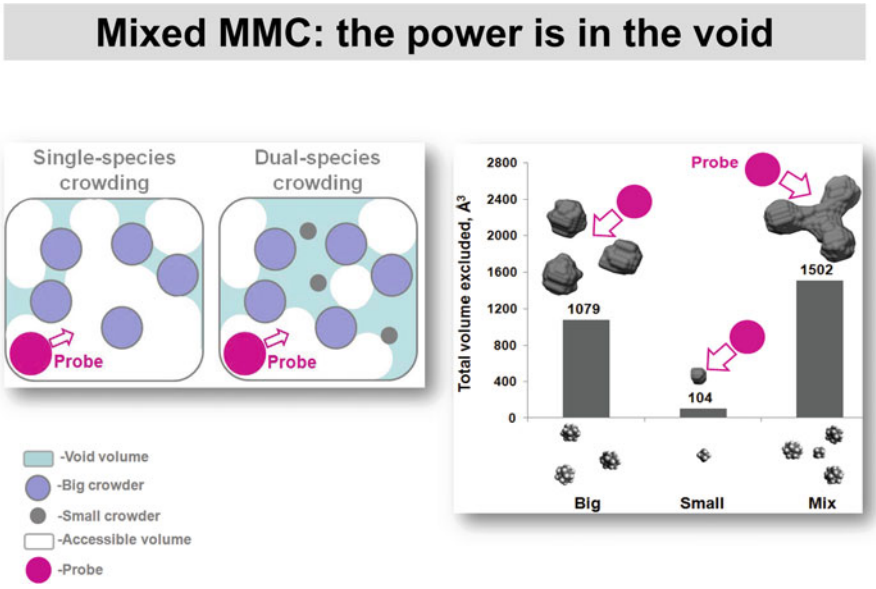


Fig. 6 Modelling of mixed macromolecular crowding. Left: Comparison to the volume that is accessible to a probe in a volume that is occupied by one species of crowders (single) or two different species (dual). In the mixed condition, the small crowder does not occupy a significant volume but reduces the average distance between larger crowders to a value lower than the probe diameter: a void volume is created. The total excluded volume is therefore greater than the sum of the crowders' volumes, as the small crowder is granted with extra volume occupancy (the void volume) due to its proximity to the big crowders. Right: molecular modeling reveals that the total void volume generated by mixed MMC is larger than the sum of void volumes generated by either crowding component. Thus, mixing crowders is a powerful approach to tune an aqueous system. (Reprinted with permission from Dewavrin et al. 2015. Copyright (2015) American Chemical Society)

cooler environment (Yang et al. 2005; Yamato and Okano 2004). This feat is achieved by coating tissue culture polystyrene with poly (N-isopropylacrylamide) (variously abbreviated PNIPAAm). At a certain temperature, such as 37 °C, PNIPAAm strands are coiled up, but at lower temperatures, like 20 °C, the coils unwind. Cell seeded onto PNIPAAm would find a smooth adhesive surface at 37 °C and then would find themselves pushed away from underneath as the polymer uncoils when the culture medium cools down outside of the incubator (Yamato et al. 2007). The chemistry behind this material is based on its glass transition temperature (Rollason et al. 1993; von Recum et al. 1998). In conditions of low ECM deposition, these thermosensitive polymers work well. However, under macromolecular crowding conditions, where abundant ECM is deposited, copolymers of PNIPAAm are necessary to detach intact and ECM-rich tissue equivalents (Satyam et al. 2014).

8 Impact of Macromolecular Crowding on Three-Dimensional Extracellular Matrix Networks and Associated Cell Signaling Events

2D and 3D in vitro systems are extensively used in tissue engineering and regenerative medicine (Shologu et al. 2016; Li and Kilian 2015). Macromolecular crowding contributes to a blurring of the boundaries between what is called "2D" and "3D" cell culture. Traditionally, the term 3D tissue engineering is used to describe thick, tissue-like constructs, often cells seeded onto a scaffold. We posit that there is a clear Z-dimension (height) in rich in ECM, scaffold-free, crowded cultures, which better imitates the in vivo-like cell microenvironment without the shortcomings that typical 3D systems present in terms of microscopic analysis and viability assessment.

Confocal imaging analysis suggested that adipocytes generated from mesenchymal stem cells under MMC were covered and embedded in ECM, resembling a layered system with cells embedded between layers of ECM (Lee et al. 2016; Bolevich et al. 2016) (Fig. 7). With more collagen available around them, due to macromolecular crowding, the cells can generate more interfaces with the ECM and especially more attachment points, even on the top of the cells. This was graphically demonstrated by the presence of focal adhesions signifying an increased cell-matrix engagement under crowding conditions (Lee et al. 2016). With more focal adhesions engaging a richer ECM, the integrin-mediated signaling is likely to increase and even change, as cells are integrative systems which constantly process and combine various extracellular signals to adapt their behavior in response to external stimuli. This three-dimensional architecture of the ECM technology has also enabled investigations into the intrinsic brown potential of adipogenic differentiation of bone marrow-derived stem cells and progenitors from the stromal vascular fraction of subcutaneous fat (Lee et al. 2016). A similar 3D ECM network surrounding adipocytes has been observed in vivo by electron microscopy of native adipose tissue (Flynn 2010; Sbarbati et al. 1987). In vitro, this situation is usually found only in cells embedded in artificially created 3D matrices (Polacheck et al. 2014). This means that a monolayer culture system can be turned around into an ultra-flat 3D system via macromolecular crowding, a consideration that is important for the design of cell-based assays for the development of drug screening tests.

As we have seen, MMC can induce a variety of cells to deposit much more ECM than under standard conditions and with higher complexity than artificial/engineered matrices. This offers the opportunity to develop these rich in ECM cell constructs as an optimal niche for in vitro expansion of various cell types, following decellularization. For example, human embryonic stem cells benefit from a human fibroblast matrix deposited under crowding conditions and can be passaged effectively on such cell-produced matrix (Peng et al. 2012). The most recent example of the successful application of MMC was the generation of (decellularized) bone marrow matrix for the cultivation of hematopoietic stem cells, applying mixed MMC with novel crowder combinations (Prewitz et al. 2015). The application of crowding to produce the full depositional cascade of collagen in complete living

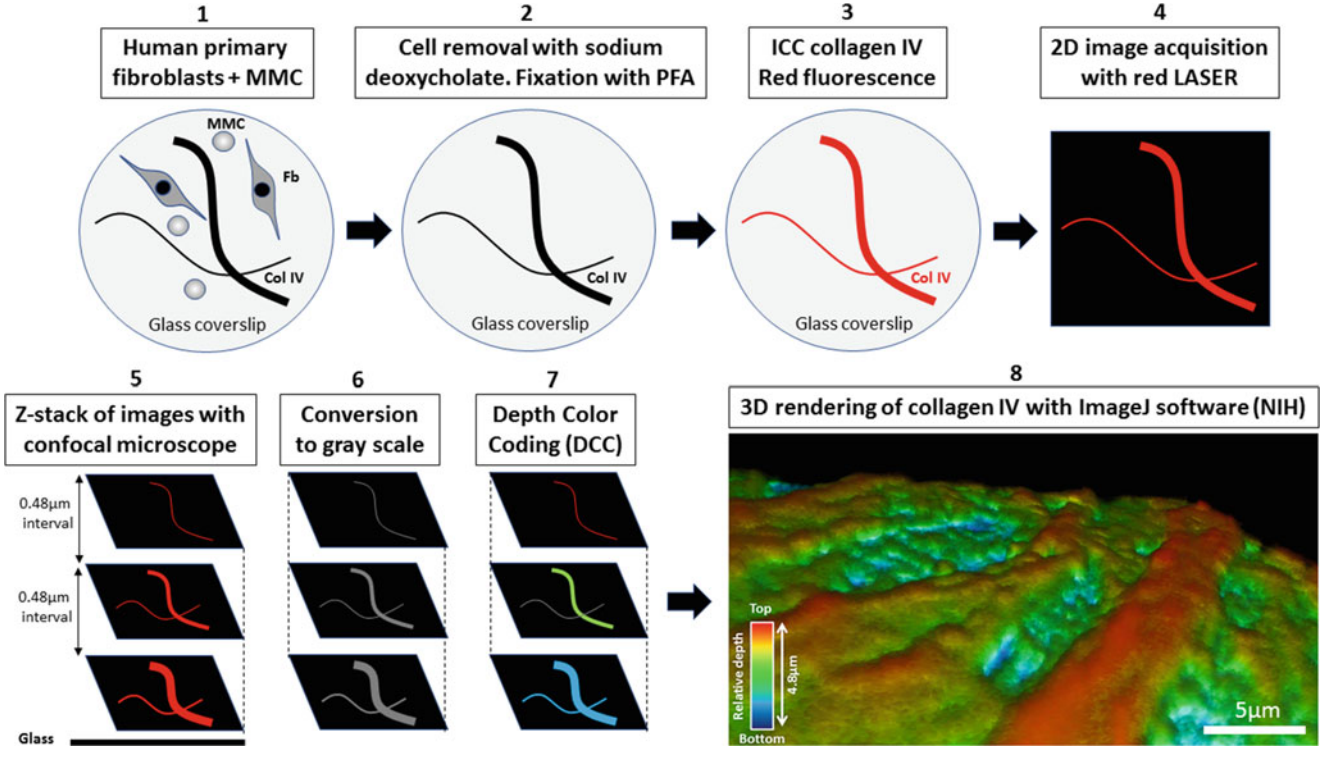


Fig. 7 3D rendering of an ECM of collagen IV produced by human primary fibroblasts upon macromolecular crowding (*MMC*). (1) Human primary fibroblasts (*Fb*) were seeded on glass coverslips and incubated in the presence of crowders (MMC) resulting in an enhanced deposition of collagen IV (*Col IV*). (2) The cells were removed with sodium deoxycholate. The samples were then washed and fixed with PFA. (3) Collagen IV was stained by immunocytochemistry (*ICC*). (4) The samples were then imaged in 2D using a red LASER. (5) The samples were then imaged plane by plane (Z-stack) using an inverted confocal microscope

fibroblast monolayers has led to the construction of a high content screening tool, the scar in a jar, for antifibrotic compound screening (Chen et al. 2009; Chen and Raghunath 2009).

9 Conclusion

Cells in tissue culture media basically represent seawater with some additional ingredients including a fraction of fetal calf serum. This makes for a very dilute environment when compared with the crowded intra- and extracellular milieus. This ocean of dilution leads to a number of biochemical and biophysical processes to occur with less speed and efficiency than they would in vivo. It is often claimed that in vitro cell culture is artificial to start with and therefore is of limited physiological relevance. While this argument is often used to make a case for animal experimentation, it should not be an excuse for not improving cell culture conditions. We believe that much can be done to move in vitro cell culture systems closer to human physiology by correcting some deficiencies, such as too much oxygen, lack of appropriate surface signals, the absence of biomechanical stimulation and macromolecular crowding, and a deficient microenvironment. It is worthwhile considering that the need for 3D tissue-engineered systems, living organ equivalents, is increasing in industry. There is societal pressure to abandon animal experimentation which is increasingly translating into political pressure and, therefore, legislation to enforce the 3R of animal welfare. Within this framework, tissue-engineered tissue equivalents will see a continued demand as long as they are robust and can be validated. In this context, macromolecular crowding can be a very valuable tool for not only answering fundamental questions but also increasing the functionality of cells in vitro by facilitating faster and deeper differentiation of progenitor cells or greater phenotypic stabilization, all via the generation of a complex human ECM.

Acknowledgments This work was supported from the Health Research Board; Health Research Awards Programme, under the grant agreement number HRA_POR/2011/84; and the Science Foundation Ireland, under the grant agreement number 15/CDA/3629. This publication has also been supported from the Science Foundation Ireland and the European Regional Development Fund, under the grant agreement number 13/RC/2073. The authors would also like to acknowledge the Tendon Therapy Train, H2020-MSCA-ITN-2015 Programme, grant agreement number 676338. M.R. acknowledges the support of start-up funding from the Zurich University of Applied Sciences

Fig. 7 (continued) (Zeiss LSM510) with an interval of 0.48 μm between two successive optical planes. (6) All images were converted into gray scale preserving pixel intensity but removing the original colors. (7) Each image was given an artificial color according to its position in the Z (vertical) axis (Depth Color Coding, *DCC*). Images acquired at the bottom of the sample (near the glass coverslip) were colored in blue. Images acquired on the top of the sample were artificially colored in red. (8) The stack of color-coded pictures was then imported into ImageJ software (NIH) for 3D rendering. (For more information on the Material and Methods, refer to Benny et al. 2015, PMID: 25058150)

(9710.3.13.5.0146.01) and Biotechnet (NTN Swiss Biotech) support via the competence Center TEDD (Tissue Engineering for Drug Development and Substance Testing) and acknowledges membership in the EU COST Action Programme CellFit CA16119.

References

Ahmed N (2005) Advanced glycation endproducts – role in pathology of diabetic complications. Diabetes Res Clin Pract 67(1):3–21

Alford AI, Hankenson KD (2006) Matricellular proteins: extracellular modulators of bone development, remodeling, and regeneration. Bone 38(6):749–757

Ang XM, Lee MH, Blocki A, Chen C, Ong LS, Asada HH, Sheppard A, Raghunath M (2013) Macromolecular crowding amplifies adipogenesis of human bone marrow-derived mesenchymal stem cells by enhancing the pro-adipogenic microenvironment. Tissue Eng A 20:966–981

Ang XM, Lee MH, Blocki A, Chen C, Ong LL, Asada HH, Sheppard A, Raghunath M (2014) Macromolecular crowding amplifies adipogenesis of human bone marrow-derived mesenchymal stem cells by enhancing the pro-adipogenic microenvironment. Tissue Eng A 20(5–6):966–981

Aplin A, Howe A, Alahari S, Juliano R (1998) Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol Rev 50(2):197–264

Arigony ALV, de Oliveira IM, Machado M, Bordin DL, Bergter L, Prá D, Pêgas Henriques JA (2013) The influence of micronutrients in cell culture: a reflection on viability and genomic stability. Biomed Res Int 2013:597282

Barnes M (1975) Function of ascorbic acid in collagen metabolism. Ann N Y Acad Sci 258(1):264–277

Barnes D, Sato G (1980) Serum-free cell culture: a unifying approach. Cell 22(3):649–655

Bateman JF, Golub SB (1990) Assessment of procollagen processing defects by fibroblasts cultured in the presence of dextran sulphate. Biochem J 267:573–577

Bateman JF, Cole WG, Pillow JJ, Ramshaw J (1986) Induction of procollagen processing in fibroblast cultures by neutral polymers. J Biol Chem 261(9):4198–4203

Benecke B-J, Ben-Ze'ev A, Penman S (1978) The control of mRNA production, translation and turnover in suspended and reattached anchorage-dependent fibroblasts. Cell 14(4):931–939

Benny P, Badowski C, Lane EB, Raghunath M (2015) Making More Matrix: Enhancing the deposition of dermal-epidermal junction components in vitro and accelerating organotypic skin culture development, using macromolecular crowding, Tissue Engineering A, 21 (1–2):183–192

Bettger WJ, McKeehan WL (1986) Mechanisms of cellular nutrition. Physiol Rev 66(1):1–35

Birk DE, Fitch J, Babiarz J, Doane K, Linsenmayer T (1990) Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J Cell Sci 95(4):649–657

Birnie J, Grayson J (1952) Observations on temperature distribution and liver blood flow in the rat. J Physiol 116(2):189

Bolevich S, Kogan AH, Zivkovic V, Djuric D, Novikov AA, Vorobyev SI, Jakovljevic V (2016) Protective role of carbon dioxide (CO_2) in generation of reactive oxygen species. Mol Cell Biochem 411(1–2):317–330

Bonnans C, Chou J, Werb Z (2014) Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 15(12):786–801

Bornstein P (1974) The structure and assembly of procollagen – a review. J Supramol Struct 2(2–4):108–120

Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414(6865):813–820

Cetta G, Rossi A, Tenni R, Valli M, Forlino A, Zanaboni G, Dyne K, Burgio GR (1993) Deposition of mutant type I collagen in the extracellular matrix of cultured dermal fibroblasts in osteogenesis imperfecta. Connect Tissue Res 29(1):41–49

Chen CZ, Raghunath M (2009) Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis state of the art. Fibrogenesis Tissue Repair 2:7

Chen SS, Fitzgerald W, Zimmerberg J, Kleinman HK, Margolis L (2007) Cell-cell and cell-extracellular matrix interactions regulate embryonic stem cell differentiation. Stem Cells 25(3):553–561

Chen CZ, Peng YX, Wang ZB, Fish PV, Kaar JL, Koepsel RR, Russell AJ, Lareu RR, Raghunath M (2009) The scar-in-a-jar: studying potential antifibrotic compounds from the epigenetic to extracellular level in a single well. Br J Pharmacol 158(5):1196–1209

Chen C, Loe F, Blocki A, Peng Y, Raghunath M (2011) Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies. Adv Drug Deliv Rev 63(4):277–290

Cigognini D, Lomas A, Kumar P, Satyam A, English A, Azeem A, Pandit A, Zeugolis D (2013) Engineering in vitro microenvironments for cell based therapies and drug discovery. Drug Discov Today 18(21–22):1099–1108

Cigognini D, Gaspar D, Kumar P, Satyam A, Alagesan S, Sanz-Nogués C, Griffin M, O'Brien T, Pandit A, Zeugolis DI (2016) Macromolecular crowding meets oxygen tension in human mesenchymal stem cell culture – a step closer to physiologically relevant in vitro organogenesis. Sci Rep 6:30746. https://doi.org/10.1038/srep30746

Collin EC, Grad S, Zeugolis DI, Vinatier CS, Clouet JR, Guicheux JJ, Weiss P, Alini M, Pandit AS (2011) An injectable vehicle for nucleus pulposus cell-based therapy. Biomaterials 32(11):2862–2870

Curtis A, Forrester J, McInnes C, Lawrie F (1983) Adhesion of cells to polystyrene surfaces. J Cell Biol 97(5):1500–1506

Damsky C (1999) Extracellular matrix–integrin interactions in osteoblast function and tissue remodeling. Bone 25(1):95–96

Dewavrin JY, Hamzavi N, Shim VP, Raghunath M (2014) Tuning the architecture of three-dimensional collagen hydrogels by physiological macromolecular crowding. Acta Biomater 10(10):4351–4359

Dewavrin JY, Abdurrahiem M, Blocki A, Musib M, Piazza F, Raghunath M (2015) Synergistic rate boosting of collagen fibrillogenesis in heterogeneous mixtures of crowding agents. J Phys Chem B 119(12):4350–4358

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317

Eagle H (1955) Nutrition needs of mammalian cells in tissue culture. Science 122:501–504

Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci 26(10):597–604

Fleischmajer R, Perlish J, Burgeson R, Shaikh-Bahai F, Timpl R (1990) Type I and type III collagen interactions during fibrillogenesis. Ann N Y Acad Sci 580(1):161–175

Flynn LE (2010) The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. Biomaterials 31(17):4715–4724

Gelse K, Pöschl E, Aigner T (2003) Collagens – structure, function and biosynthesis. Adv Drug Deliv Rev 55(12):1531–1546

Graham MF, Willey A, Adams J, Yager D, Diegelmann RF (1995) Role of ascorbic acid in procollagen expression and secretion by human intestinal smooth muscle cells. J Cell Physiol 162(2):225–233

Gregoire FM, Smas CM, Sul HS (1998) Understanding adipocyte differentiation. Physiol Rev 78(3):783–809

Grinnell F, Feld MK (1979) Initial adhesion of human fibroblasts in serum-free medium: possible role of secreted fibronectin. Cell 17(1):117–129

Gstraunthaler G, Lindl T, van der Valk J (2013) A plea to reduce or replace fetal bovine serum in cell culture media. Cytotechnology 65(5):791–793

Harve KS, Raghunath M, Lareu RR, Rajagopalan R (2006) Macromolecular crowding in biological systems: dynamic light scattering (DLS) to quantify the excluded volume effect (EVE). Biophys Rev Lett 1(3):317–325

Harve KS, Lareu R, Rajagopalan R, Raghunath M (2010) Understanding how the crowded interior of cells stabilizes DNA/DNA and DNA/RNA hybrids–in silico predictions and in vitro evidence. Nucleic Acids Res 38(1):172–181

Hayman EG, Pierschbacher MD, Suzuki S, Ruoslahti E (1985) Vitronectin – a major cell attachment-promoting protein in fetal bovine serum. Exp Cell Res 160(2):245–258

Hoffman W, Charbel F, Gonzalez Portillo G, Edelman G, Ausman J (1998) Regional tissue pO_2 pCO_2, pH and temperature measurement. Neurol Res 20(sup1):S81–S84

Hojima Y, Behta B, Romanic AM, Prockop DJ (1994) Cleavage of type I procollagen by *C*- and *N*-proteinases is more rapid if the substrate is aggregated with dextran sulfate or polyethylene glycol. Anal Biochem 223(2):173–180

Honn KV, Singley JA, Chavin W (1975) Fetal bovine serum: a multivariate standard. Proc Soc Exp Biol Med 149(2):344–347

Jukkola A, Risteli J, Risteli L (1991) Effect of dextran on synthesis, secretion and deposition of type III procollagen in cultured human fibroblasts. Biochem J 279:49–54

Kadler KE, Holmes DF, Trotter JA, Chapman JA (1996) Collagen fibril formation. Biochem J 316(Pt 1):1–11

Kielty CM, Grant ME (2003) The collagen family: structure, assembly, and organization in the extracellular matrix. In: Connective tissue and its heritable disorders. Wiley, Hoboken, pp 159–221

Kiple KF (2002) The Caribbean slave: a biological history. Cambridge University Press, Cambridge

Kumar P, Satyam A, Fan X, Collin E, Rochev Y, Rodriguez BJ, Gorelov A, Dillon S, Joshi L, Raghunath M, Pandit A, Zeugolis DI (2015a) Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. Sci Rep 5:8729. https://doi.org/10.1038/srep08729

Kumar P, Satyam A, Fan X, Rochev Y, Rodriguez BJ, Gorelov A, Joshi L, Raghunath M, Pandit A, Zeugolis DI (2015b) Accelerated development of supramolecular corneal stromal-like assemblies from corneal fibroblasts in the presence of macromolecular crowders. Tissue Eng Part C 21(7):660–670

Kumar P, Satyam A, Cigognini D, Pandit A, Zeugolis DI (2018) Low oxygen tension and macromolecular crowding accelerate extracellular matrix deposition in human corneal fibroblast culture. J Tissue Eng Regen Med 12:6–18

Kyburz KA, Anseth KS (2015) Synthetic mimics of the extracellular matrix: how simple is complex enough? Ann Biomed Eng 43(3):489–500

Lareu RR, Subramhanya KH, Peng Y, Benny P, Chen C, Wang Z, Rajagopalan R, Raghunath M (2007a) Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged macromolecules: the biological relevance of the excluded volume effect. FEBS Lett 581(14):2709–2714

Lareu RR, Arsianti I, Subramhanya HK, Yanxian P, Raghunath M (2007b) In vitro enhancement of collagen matrix formation and crosslinking for applications in tissue engineering: a preliminary study. Tissue Eng 13(2):385–391

Lee PJ, Choi AM (2003) Pathways of cell signaling in hyperoxia. Free Radic Biol Med 35(4):341–350

Lee S, Solow-Cordero DE, Kessler E, Takahara K, Greenspan DS (1997) Transforming growth factor-beta regulation of bone morphogenetic protein-1/procollagen C-proteinase and related proteins in fibrogenic cells and keratinocytes. J Biol Chem 272(30):19059–19066

Lee MH, Goralczyk AG, Kriszt R, Ang XM, Badowski C, Li Y, Summers SA, Toh SA, Yassin MS, Shabbir A, Sheppard A, Raghunath M (2016) ECM microenvironment unlocks brown adipogenic potential of adult human bone marrow-derived MSCs. Sci Rep 6:21173

Li Y, Kilian K (2015) Bridging the gap: from 2D cell culture to 3D microengineered extracellular matrices. Adv Healthc Mater 4(18):2780–2796
Lilla J, Stickens D, Werb Z (2002) Metalloproteases and adipogenesis: a weighty subject. Am J Pathol 160(5):1551–1554
Lin CQ, Bissell MJ (1993) Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J 7(9):737–743
Lodish H, Berk A, Zipursky S, Matsudaira P, Baltimore D, Darnell J (2000) Collagen: the fibrous proteins of the matrix. In: Molecular cell biology. W. H. Freeman, New York
Lutolf M, Hubbell J (2005) Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 23(1):47–55
Magno V, Friedrichs J, Weber HM, Prewitz MC, Tsurkan MV, Werner C (2017) Macromolecular crowding for tailoring tissue-derived fibrillated matrices. Acta Biomater 55:109–119
Mather JP, Roberts PE (1998) The physical environment. In: Introduction to cell and tissue culture: theory and technique. Springer Science & Business Media, Boston, pp 25–41
McDowell L (2013) Vitamin history, the early years, 1st edn. Design eBook Publishing. http://firsteditiondesignpublishing.com/html/index.html
Minton AP (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol Chem 276(14):10577–10580
Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A (2010) Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7(2):150–161
Mott JD, Thomas CL, Rosenbach MT, Takahara K, Greenspan DS, Banda MJ (2000) Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. J Biol Chem 275(2):1384–1390
Mouw JK, Ou G, Weaver VM (2014) Extracellular matrix assembly: a multiscale deconstruction. Nat Rev Mol Cell Biol 15(12):771–785
Murad S, Grove D, Lindberg K, Reynolds G, Sivarajah A, Pinnell S (1981) Regulation of collagen synthesis by ascorbic acid. Proc Natl Acad Sci 78(5):2879–2882
Nakajima I, Aso H, Yamaguchi T, Ozutsumi K (1998) Adipose tissue extracellular matrix: newly organized by adipocytes during differentiation. Differentiation 63(4):193–200
Niles WD, Coassin PJ (2008) Cyclic olefin polymers: innovative materials for high-density multiwell plates. Assay Drug Dev Technol 6(4):577–590
Notbohm H, Mosler S, Bodo M, Yang C, Lehmann H, Bätge B, Müller PK (1992) Comparative study on the thermostability of collagen I of skin and bone: influence of posttranslational hydroxylation of prolyl and lysyl residues. J Protein Chem 11(6):635–643
Owens MR, Cimino CD (1982) Synthesis of fibronectin by the isolated perfused rat liver. Blood 59(6):1305–1309
Park D, Lim J, Park JY, Lee S-H (2015) Concise review: stem cell microenvironment on a chip: current technologies for tissue engineering and stem cell biology. Stem Cells Transl Med 4(11):1352–1368
Peng YX, Raghunath M (2010) Learning from Nature. Emulating macromolecular crowding to drive extracellular matrix enhancement for the creation of connective tissue in vitro. Source: Tissue Engineering, Book edited by: Daniel Eberli MD PhD, ISBN: 978-953-307-079-7, Publisher: INTECH, Publishing date: March 2010
Peng Y, Bocker MT, Holm J, Toh WS, Hughes CS, Kidwai F, Lajoie GA, Cao T, Lyko F, Raghunath M (2012) Human fibroblast matrices bio-assembled under macromolecular crowding support stable propagation of human embryonic stem cells. J Tissue Eng Regen Med 6(10):e74–e86
Peterkofsky B (1991) Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 54(6):1135S–1140S
Polacheck WJ, German AE, Mammoto A, Ingber DE, Kamm RD (2014) Mechanotransduction of fluid stresses governs 3D cell migration. Proc Natl Acad Sci 111(7):2447–2452
Prewitz MC, Stißel A, Friedrichs J, Träber N, Vogler S, Bornhäuser M, Werner C (2015) Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding. Biomaterials 73:60–69
Raghunath M, Bruckner P, Steinmann B (1994) Delayed triple helix formation of mutant collagen from patients with osteogenesis imperfecta. J Mol Biol 236(3):940–949

Raghunath M, Hopfner B, Aeschlimann D, Luthi U, Meuli M, Altermatt S, Gobet R, Bruckner-Tuderman L, Steinmann B (1996) Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyte autografts. Anchoring fibrils are a target for tissue transglutaminase. J Clin Invest 98(5):1174–1184

Raghunath M, Unsold C, Kubitscheck U, Bruckner-Tuderman L, Peters R, Meuli M (1998) The cutaneous microfibrillar apparatus contains latent transforming growth factor-beta binding protein-1 (LTBP-1) and is a repository for latent TGF-beta1. J Investig Dermatol 111(4):559–564

Raghunath M, Cankay R, Kubitscheck U, Fauteck JD, Mayne R, Aeschlimann D, Schlotzer-Schrehardt U (1999) Transglutaminase activity in the eye: cross-linking in epithelia and connective tissue structures. Invest Ophthalmol Vis Sci 40(12):2780–2787

Ramsey W, Hertl W, Nowlan E, Binkowski N (1984) Surface treatments and cell attachment. In Vitro 20(10):802–808

Rashid R, Lim NSJ, Chee SML, Png SN, Wohland T, Raghunath M (2014) Novel use for polyvinylpyrrolidone as a macromolecular crowder for enhanced extracellular matrix deposition and cell proliferation. Tissue Eng Part C Methods 20(12):994–1002

Ratcliffe A, Niklason LE (2002) Bioreactors and bioprocessing for tissue engineering. Ann N Y Acad Sci 961(1):210–215

Rollason G, Davies JE, Sefton MV (1993) Preliminary report on cell culture on a thermally reversible copolymer. Biomaterials 14(2):153–155

Royce PM, Steinmann B (2003) Connective tissue and its heritable disorders: molecular, genetic, and medical aspects. Wiley, New York

Ruoslahti E (1996) RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 12(1):697–715

Satyam A, Kumar P, Fan X, Gorelov A, Rochev Y, Joshi L, Peinado H, Lyden D, Thomas B, Rodriguez B, Raghunath M, Pandit A, Zeugolis D (2014) Macromolecular crowding meets tissue engineering by self-assembly: a paradigm shift in regenerative medicine. Adv Mater 26(19):3024–3034

Satyam A, Kumar P, Cigognini D, Pandit A, Zeugolis DI (2016) Low, but not too low, oxygen tension and macromolecular crowding accelerate extracellular matrix deposition in human dermal fibroblast culture. Acta Biomater 44:221–231

Sbarbati A, Zancanaro C, Cigolini M, Cinti S (1987) Brown adipose tissue: a scanning electron microscopic study of tissue and cultured adipocytes. Acta Anat (Basel) 128(1):84–88

Selvarajan S, Lund LR, Takeuchi T, Craik CS, Werb Z (2001) A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation. Nat Cell Biol 3(3):267–275

Sholoqu N, Szegezdi E, Lowery A, Kerin M, Pandit A, Zeugolis D (2016) Recreating complex pathophysiologies in vitro with extracellular matrix surrogates for anticancer therapeutics screening. Drug Discov Today 21(9):1521–1531

Shoulders MD, Raines RT (2009) Collagen structure and stability. Annu Rev Biochem 78:929–958

Stenman S, Vaheri A (1978) Distribution of a major connective tissue protein, fibronectin, in normal human tissues. J Exp Med 147(4):1054–1064

Taipale J, Saharinen J, Hedman K, Keski-Oja J (1996) Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. J Histochem Cytochem 44(8):875–889

Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK (2016) Extracellular matrix structure. Adv Drug Deliv Rev 97:4–27

Trackman PC (2005) Diverse biological functions of extracellular collagen processing enzymes. J Cell Biochem 96(5):927–937

Van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker F, Prieto P (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicol In Vitro 18(1):1–12

Van der Valk J, Brunner D, De Smet K, Svenningsen ÅF, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino M (2010) Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods. Toxicol In Vitro 24(4):1053–1063

von Recum H, Kikuchi A, Okuhara M, Sakurai Y, Okano T, Kim SW (1998) Retinal pigmented epithelium cultures on thermally responsive polymer porous substrates. J Biomat Sci Polym Ed 9(11):1241–1253

Wang Z, Griffin M (2012) TG2, a novel extracellular protein with multiple functions. Amino Acids 42(2–3):939–949

Werner J, Buse M (1988) Temperature profiles with respect to inhomogeneity and geometry of the human body. J Appl Physiol 65(3):1110–1118

Woolridge E, Turchi SL, Edwards JR (1986) The peroxidase-glucose oxidase enzyme system in the undergraduate laboratory. Biochem Educ 14(2):82–83

Yamato M, Okano T (2004) Cell sheet engineering. Mater Today 7(5):42–47

Yamato M, Akiyama Y, Kobayashi J, Yang J, Kikuchi A, Okano T (2007) Temperature-responsive cell culture surfaces for regenerative medicine with cell sheet engineering. Prog Polym Sci 32(8):1123–1133

Yamauchi M, Sricholpech M (2012) Lysine post-translational modifications of collagen. Essays Biochem 52:113–133

Yang J, Yamato M, Kohno C, Nishimoto A, Sekine H, Fukai F, Okano T (2005) Cell sheet engineering: recreating tissues without biodegradable scaffolds. Biomaterials 26(33):6415–6422

Zeiger AS, Loe FC, Li R, Raghunath M, Van Vliet KJ (2012) Macromolecular crowding directs extracellular matrix organization and mesenchymal stem cell behavior. PLoS One 7(5):e37904

Zeugolis DI, Panengad PP, Yew ES, Sheppard C, Phan TT, Raghunath M (2010) An in situ and in vitro investigation for the transglutaminase potential in tissue engineering. J Biomed Mater Res A 92(4):1310–1320

Zheng X, Baker H, Hancock WS, Fawaz F, McCaman M, Pungor E (2006) Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. Biotechnol Prog 22(5):1294–1300

Zhou H-X (2008) Effect of mixed macromolecular crowding agents on protein folding. Proteins 72(4):1109–1113

Zhou HX, Rivas G, Minton AP (2008) Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. Annu Rev Biophys 37:375–397

microRNA Modulation

Irene Mencía Castaño, Rosanne M. Raftery, Caroline M. Curtin, Johannes Grillari, Heinz Redl, and Fergal J. O'Brien

Contents

I. Mencía Castaño · R. M. Raftery · C. M. Curtin · F. J. O'Brien (✉)
Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland

Advanced Materials and Bioengineering Research Centre (AMBER), RCSI and TCD, Dublin, Ireland
e-mail: imenciacastano@rcsi.ie; rosanneraftery@rcsi.ie; carolinecurtin@rcsi.ie; fjobrien@rcsi.ie

J. Grillari
Department of Biotechnology, BOKU - University of Natural Resources and Life Sciences Vienna, Vienna, Austria
e-mail: johannes.grillari@boku.ac.at

H. Redl
AUVA Trauma Research Center, Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria
e-mail: office@trauma.lbg.ac.at

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_34

Abstract

microRNAs are small and evolutionary conserved RNA molecules that vastly fine-tune protein expression at a posttranscriptional level. microRNA modulation has recently surfaced as powerhouse feeding the progress of novel strategies for tissue and cell engineering and regeneration. The field is growing exponentially each year and approaching clinical applications, with considerable progress in identifying biomarkers for personalized medical needs and also harnessing the therapeutic potential of these molecules to finely enhance tissue repair. This chapter aims to support beginner and expert researchers alike to delve into this emerging dynamic field. Within this chapter, we provide an overview of the biology and function of microRNAs and how they are being addressed within the tissue engineering and regenerative medicine (TERM) arena in terms of resources, applications, and development projection. Specific attention is given to the advances in the development of specialized delivery systems for microRNAs, which largely involves the application of biomaterial scaffolds, and to finalize, we review the proven therapeutic potential of microRNAs to date within the TERM space. Overall, this chapter underlines the exciting potential of microRNA modulation for cell engineering and regeneration.

1 Introduction to microRNA Biology for Cell Engineering and Regeneration

Novel approaches to engineer cells are currently gaining much traction in the field of tissue engineering and regenerative medicine (TERM) (Yau et al. 2012). These innovative strategies represent a step beyond the traditional application of cell-based therapy – with unmodified cells, biomaterial-based therapy, and the combination of both elements (Khademhosseini et al. 2009; Evans 2013). The main aim of applying cell engineering to TERM is to enhance the ability of the target cells to

regenerate tissues and re-establish a healthy function (Nerem 1991); these target cells can be part of cell therapies or be engineered directly at the host's tissue defect site. To translate the successful cell engineering methods into therapies applicable to TERM, the cross-functional collaboration of bioengineering, molecular biology, biopharmaceutics, materials science, nanotechnology, and medical/clinical experts will remain key. microRNAs represent a recent breakthrough in molecular biology, and with the understanding of how these small RNAs can influence tissue regeneration, microRNA modulation shines through as a novel promising route for cell engineering. In addition, microRNA profiling and biomaterials research has begun to merge toward the development of physiologically relevant in vitro 3D models, providing game-changing insights on the transcriptomic modulation of tissue repair (Cui et al. 2014). Key concepts of the origin and potential of microRNAs and microRNA therapeutics, including the methods and resources suitable for their investigation, will be discussed in this chapter. A specific focus will be on the state-of-the-art research on cell engineering and regeneration using biomaterials to deliver microRNA therapeutics, as well as on the hurdles for the clinical translation of these technologies.

1.1 microRNAs as Part of the Natural Cellular Mechanism of RNA Interference

microRNAs (miRNAs) have positioned themselves at the top of the natural RNA interference tools for cell engineering applications, thus challenging the 1950s dogma of hierarchical gene expression, which postulates that oligonucleotide sequences only act as units of information storage (Rinn and Chang 2012). The discovery of miRNAs led to the realization that oligonucleotides may also convey enzymatic and regulatory activities to manage the use of the very information stored within and ultimately allow the synthesis of proteins and its control (Pearson 2006). These activities entail a broad spectrum of RNA molecules such as ribosomal (r)RNAs, transfer (t)RNAs, small nuclear (sn)RNAs, small nucleolar (sno)RNAs, noncoding (nc)RNAs, or RNA interference (RNAi) (Fig. 1; Alberts et al. 2002). Ultimately, controlling the synthesis of proteins by RNAi presents an advanced and very valuable cell engineering strategy (Hackl et al. 2011).

Although miRNAs are involved in the RNA interference (RNAi) process, Fire et al. first established this process in *C. elegans* years before miRNAs were discovered (Fire et al. 1998). This process is also known as the RNA-induced silencing pathway and is initiated by long double-stranded (ds)RNA oligonucleotides. Such long dsRNA molecules can be either naturally generated or introduced exogenously as an intermediate of viral replication (Ketting et al. 1999; Mourrain et al. 2000; Li et al. 2002; Aravin et al. 2003) or via experimental gene knockdown (Fire et al. 1998). In the RNAi process, the dsRNA is processed by Dicer into smaller RNAs (Hamilton and Baulcombe 1999; Hammond et al. 2000; Knight and Bass 2001) that

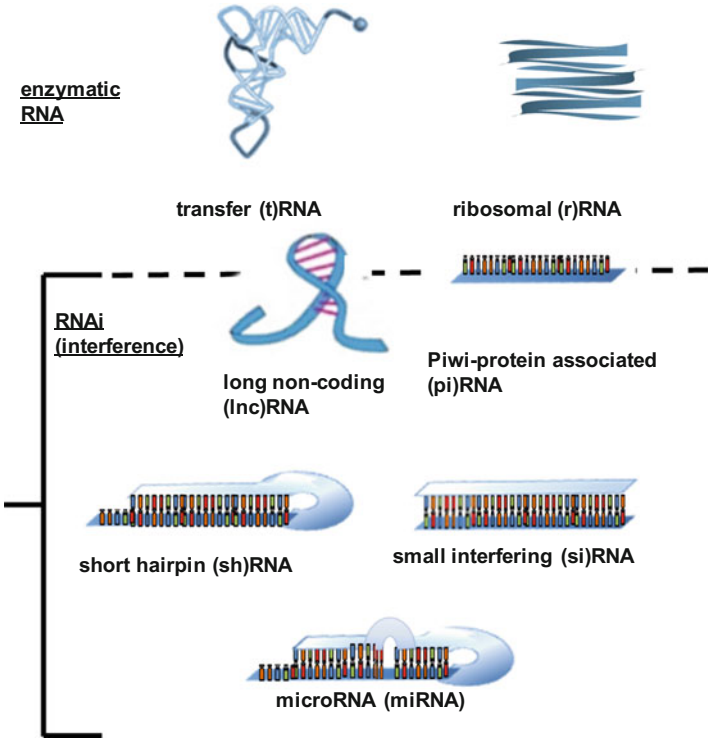


Fig. 1 Types of enzymatic RNAs that manage the expression of protein-coding genes. tRNA and rRNA cooperate in the production of protein from messenger (m)RNA as part of the translation process. This activity is subjected to the control of RNA interference (RNAi) molecules: short hairpin (sh)RNAs, small interference (si)RNAs, and microRNAs (miRNAs). Long noncoding (lnc) RNAs and Piwi-protein associated (pi)RNAs are involved in RNAi as well as in additional enzymatic functions

eventually become incorporated as single-stranded (ss)RNAs into the RNA-induced silencing complex (RISC) (Elbashir et al. 2001a; Nykanen et al. 2001; Martinez et al. 2002). The RISC is composed of the endonuclease Slicer and other structural proteins such as VIG, fragile X-related protein, and Tudor-SN (Caudy et al. 2002, 2003; Ishizuka et al. 2002). The core protein of this complex is a member of the Argonaute family with ability to bind to ssRNAs and dsRNAs through a domain denominated PAZ (Martinez et al. 2002; Hammond et al. 2001; Hutvagner and Zamore 2002; Lingel et al. 2003). Once the RISC is formed, it identifies target messenger RNAs (mRNAs) based on perfect or nearly perfect complementarity between the 3′ untranslated (UTR) region of the mRNA and 5′ terminal region of the RNAi, also known as the seed region (Elbashir et al. 2001b). This interaction directs the target mRNA for either cleavage or repression of its translation, thus resulting in either a decrease in target mRNA or protein levels (Bartel 2004).

1.2 The Discovery of microRNAs

To understand the potential of microRNAs as tools for cell engineering and regeneration, we need to review the origins of miRNA research as a field of study, starting with the original discovery of the lin-4 RNA gene in *Caenorhabditis elegans* in 1993 by Victor Ambros and colleagues, Rosalind Lee and Rhonda Feinbaum (Lee et al. 1993). This team discovered that the lin-4 gene, a known regulator of the timing in larval development, did not code for a protein but instead produced a pair of small RNAs, 61 and 22 nucleotides (nt) long, with antisense complementarity to the lin-14 gene (Wightman et al. 1993). The breakthrough was followed by the discovery, in *C. elegans*, of a second ~22 nt regulatory RNA named let-7 and the identification of let-7 homologs in the genome of human, fly, and 11 other species (Reinhart et al. 2000; Slack et al. 2000; Pasquinelli et al. 2000). Within a year, the identification of additional genes for ~22 nt noncoding RNAs surpassed 100 (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001); these genes with their RNA products were evolutionary conserved, but many functioned in particular cell types and were not related to embryonic development. To date, over 2500 miRNAs in humans have been identified (miRBase 2013), regulating up to one third of the protein-coding genome (Lewis et al. 2005). Increasing numbers of studies are discovering miRNAs that function to regulate key cellular events that are widely pursued in cell and tissue engineering. These include cell development, lineage commitment, differentiation, proliferation and apoptosis, immune response events and related diseases, tumor formation, and the progression of viral infections (Yau et al. 2012; Hu et al. 2010). From this evidence, miRNA-directed gene regulation has gained the attention of researchers in the tissue engineering and regenerative medicine arena seeking advanced solutions to restore and enhance normal tissue function.

1.3 The Biogenesis Process of microRNAs

Although miRNAs share cellular RNAi machinery with other types of dsRNA molecules, they must first be present in the cytoplasm to trigger their control of protein expression. This occurs following the natural biogenesis process of miRNAs (Fig. 2), which involves additional machinery and can be divided into three main phases of transcription, maturation, and RNA-induced silencing complex (RISC) assembly:

Transcription: In this step, long primary transcripts termed pri-miRNAs generate either from intronic regions of protein-coding genes or directly from intergenic or polycistronic miRNA genes containing stem-loop miRNA clusters (Lee et al. 2002). The enzyme involved in the intronic transcripts is always a type II RNA polymerase (pol) that also transcribes the corresponding protein-coding exons. However, for intergenic and polycistronic miRNA genes, both RNA pol type II

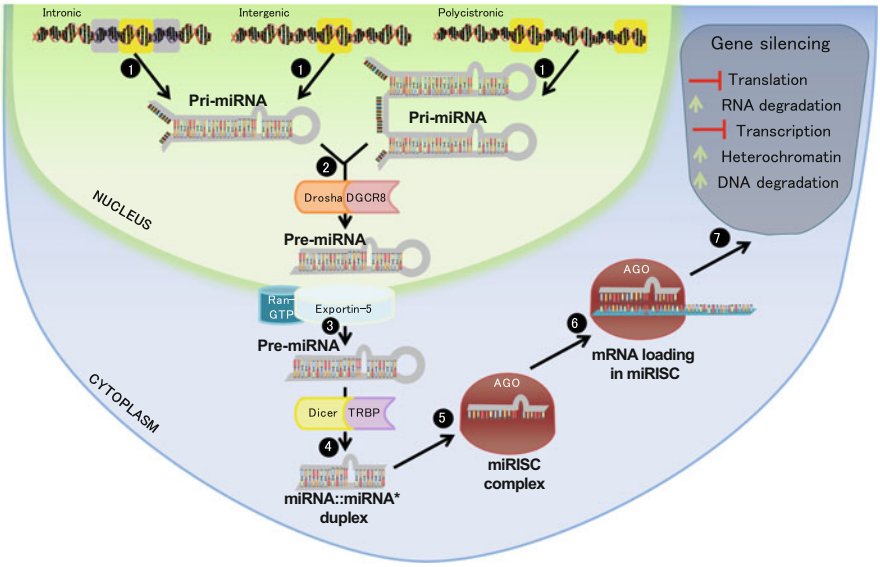


Fig. 2 miRNA biogenesis and function. (Reproduced with permission (Curtin et al. 2017). Copyright © 2017, John Wiley and Sons). (1) Transcription of miRNA genes to pri-miRNA is followed by (2) loop-processing by Drosha to generate a pre-miRNA that is then (3) transported into the cytoplasm where (4) Dicer/TRBP releases the miRNA:miRNA* duplex. (5) The mature miRNA separates to form the miRISC complex with the Argonaute (AGO) protein core, while the miRNA* strand degrades. (6) Target mRNAs interact with miRISC complexes, leading to (7) mRNA degradation or translational repression among other complex regulatory functions

and type III may carry out the transcription (Ohler et al. 2004). While RNA pol III can render efficiently processed miRNAs that function in vivo (Chen et al. 2004), RNA pol II can lead to robust expression of reporter proteins with an open reading frame downstream of intergenic miRNA genes (Johnson and Urist 1998; Johnston and Hobert 2003).

Maturation: This step is comprised of three events, two cellular locations, and several enzymes and multi-protein complexes. It starts when pri-miRNAs are cleaved in both strands leaving 60–70 nt stem-loop precursor miRNAs with 5′ phosphate and ~2 nt 3′ overhangs, defined as pre-miRNAs (Lee et al. 2002, 2003; Zeng and Cullen 2003; Basyuk et al. 2003): this occurs in the nucleus by the Drosha RNase III endonuclease. Following this, Ran-GTP and Exportin-5 actively transport the pre-miRNA into the cytoplasm (Yi et al. 2003; Lund et al. 2004). Finally, both strands of the pre-miRNA duplex are cleaved at the base of the stem-loop by the Dicer RNase III endonuclease. This process also creates 5′ phosphate and ~2 nt 3′ overhangs, generating an imperfect duplex, termed miRNA:miRNA* (Chen et al. 2004; Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). The duplex is short lived, with the two strands separating into the mature miRNA single strand and, the opposite and more unstable arm, that is, designated miRNA* or alternative strand.

RISC assembly: Finally, the single-stranded mature miRNA is selected for incorporation into the RISC, forming the miRISC complex (Mourelatos et al. 2002), while the miRNA*, detected at much lower frequencies, appears to be degraded (Aravin et al. 2003; Lim et al. 2003). Regarding the strand selection, it is hypothesized that an unknown helicase may direct the limper 5′-end strand to enter the RISC, although this must yet be fully elucidated (Khvorova et al. 2003; Schwarz et al. 2003). However, the alternative strand can be functionally active in some instances forming miRISCs, depending on the tissue, developmental, or pathophysiological state (Okamura et al. 2009). Once miRISC is formed, the interaction with the untranslated region (UTR) of the target mRNA may take place, resulting in mRNA cleavage or translational repression (Bartel 2004; Lewis et al. 2005).

The characteristic feature of miRNAs, differing from siRNAs, can be noted at the end of their biogenesis process and within a multi-protein complex. miRISC is most prone to interact with the 3′-UTR region of a target mRNA; however binding to 5′-UTR regions has also been documented (Lytle et al. 2007). Additionally, miRISC can establish both perfect and imperfect sequence complementarity binding to the mRNA targets (Guo et al. 2010). It is this partial binding that allows the multi-targeting effect of miRNAs: the seed region of a single miRNA can imperfectly bind (and thus interfere with the expression of) as many as 100 mRNAs, and the mRNA coding for a specific protein can be the subject of interaction with a collection of miRNAs (Bartel 2004; Lewis et al. 2005; Mourelatos et al. 2002; Jacobsen et al. 2013). Altogether, this overview of the known mechanism of action of miRNAs supports the increasing interest in using them as cell engineering tools to improve tissue repair.

1.4 microRNA Nomenclature

The discovery and establishment of miRNAs as a distinct class of RNA molecules necessitated the development of a uniform nomenclature system (Ambros et al. 2003; Desvignes et al. 2015; Budak et al. 2016), summarized in Table 1, to facilitate the well-ordered progress of the miRNA research field. This system is coordinated by the HUGO Gene Nomenclature Committee (HGNC) for the human miRNA genes (Gray et al. 2015) and aims to designate the different biogenic stages and variants of miRNAs as well as to keep newly discovered miRNAs generated by sequencing data and powerful bioinformatics tools in an orderly organization that would prevent overlapping in the denomination of new miRNAs (Griffiths-Jones et al. 2006). Recently, miRtrons, isomiRs, moRs, loRs, miRNA clusters, and mirror miRNAs (defined in Table 1) have been recognized as biogenic variants of miRNAs. In general, any given miRNA receives the prefix “miR” followed by a number of one to four figures and should be preceded by the three-letter code assigned to the species, i.e., *hsa-* for *Homo sapiens* (human) or *mmu-* for *Mus musculus* (mouse). The specific set of figures that identify a miRNA is set in order of discovery but also

Table 1 Nomenclature designating miRNAs and their types of interaction with target mRNAs

Term	Definition
miR-XX	miRNA-coding gene
mir-XX	Precursor hairpins (lower case)
spp – miR-XX	Mature sequence miR-XX designated for a determined species
miR-XX-1; miR-XX-2	Mature identical sequences proceeding from different genes (i.e., distinct pri-miRNA loci)
miR-XXa/b	Closely related mature sequences differing in only one or two nucleotides
miR-XX-5p/3p	Different mature miRNA sequences excised respectively from 5′- and 3′-arms of the same pre-miRNA (new nomenclature replacing miRNA and miRNA*)
miRc-XX	A miRNA cluster is a group of miRNA genes located in a small chromosomal region and subject to simultaneous regulation of their expression and thus shares a function
miRtron	Drosha-independent miR, the precursor (pre-miRNA) derives from a short intron
isomiR	A variant of posttranscriptional processing of a miRNA gene; may differ in sequence and hence in targets/functions.
moR-XX.Y	Offset RNA adjacent to the hairpin loop of the pre-miRNA, independently regulated
loR-XX	RNA single strand originating from the hairpin loop of the pre-miRNA
Mirror miR; miR-XXos	A pri-miRNA independently transcribed from both DNA strands; likely differs in seed region and hence in targets/function
miRNA::mRNA	Interaction of a miRNA and its target mRNA
6mer-1A	An exact match to positions 2–6 of the mature miRNA (the seed) followed by an adenine (“A”)
6mer	An exact match to positions 2–7 of the seed
7mer-1A	An exact match to positions 2–7 of the seed followed by an “A”
7mer-m8	An exact match to positions 2–8 of the seed + position 8
8mer-1A	An exact match to positions 2–8 of the seed + position 8 followed by an “A”

takes into account similarity or homology of the sequence with previously known miRNAs. Moreover, highly homologous miRNAs are often designated as members of “miRNA families” when they present evolutionary conserved sequences, in particular in the 5′-end region. Since the 5′-end of a miRNA contains the small nucleotide “seed region” sequence, responsible for the interaction with its mRNA targets, highly homolog seed regions between miRNAs signify that they likely share a set of mRNA targets. These miRNA families are referred to by the first discovered miRNA, i.e., the family of miR-15a/b, miR-16, miR-195, miR-424, and miR-497 is referred to as the miR-15 family. Importantly, all members of the miR-15 and miR-17 families have great impact in modulating multiple processes of interest for tissue engineering, including cell cycle, proliferation, and angiogenesis (Pekarsky et al. 2018; Porrello et al. 2011; Cimmino et al. 2005; Nunes et al. 2015; Sun et al. 2013; Caporali and Emanueli 2011; Linsley et al. 2007), which will be further described later in this chapter. Also, specific nomenclature has been established to

designate the different types of base-pairing interaction that can take place between the seed region of a miRNA and its target mRNA.

2 Project Blueprint to Harness microRNA Research for Cell Engineering

The sequence of stages necessary to develop a successful miRNA-based cell engineering strategy can be intricate for a tissue engineering researcher approaching a miRNA research project for the first time. This is because a wide range of miRNA exploration techniques have been newly created or adapted to allow their detailed study. These include bioinformatics tools, as well as techniques ranging from miRNA isolation and identification to their detection, the elucidation of their targets and the pathways behind their regulation, and ultimately their therapeutic potential. This section provides a blueprint of the resources and techniques powering the progress in this field and the standard experimental workflows designed applying these techniques.

2.1 Bioinformatic Tools in miRNA Research

There are multiple bioinformatic tools developed to allow the in silico exploration of putative functions for known miRNAs, as recently reviewed by Budak et al. (2016) and also by Akhtar and colleagues (Akhtar et al. 2016). These resources are freely available online and permit a wide range of tasks such as identification of miRNAs with their regulatory, metabolic, and signaling networks. Hence, bioinformatic analysis performed ahead of experimental studies provide highly valuable data to identify mRNA targets implicated in a particular pathway, along with the type of miRNA::mRNA target interaction taking place, its homology across species, and the region occupancy by other neighboring miRNAs (Grimson et al. 2007). Although many of these platforms offer mixed services, we can generally distinguish between target prediction tools like TargetScan (TargetScan Release 6.2 2012) or microRNA.org (cBio 2010) and compiling databases such as miRTarBase (ISBLab 2013) or MirSNP (Bhattacharya et al. 2014) (Fig. 3). The target prediction tools, programmed to predict the likelihood of miRNA::mRNA interactions, often receive vast attention in screening studies. Each of these programs is based on a different and complex algorithm that computes multiple parameters of miRNA behavior in order to yield and score a list of gene target hits (Schirle et al. 2014). These parameters include, among others, the binding free energy and subsequent thermodynamic stability of the miRNA::mRNA duplex, as well as the absence of secondary structures surrounding the seed region. Therefore, results might differ widely when comparing computational predicting tools; to narrow down the chances of false-negative or false-positive predictions, it is recommended to seek overlaps within the results pooled from multiple tools.

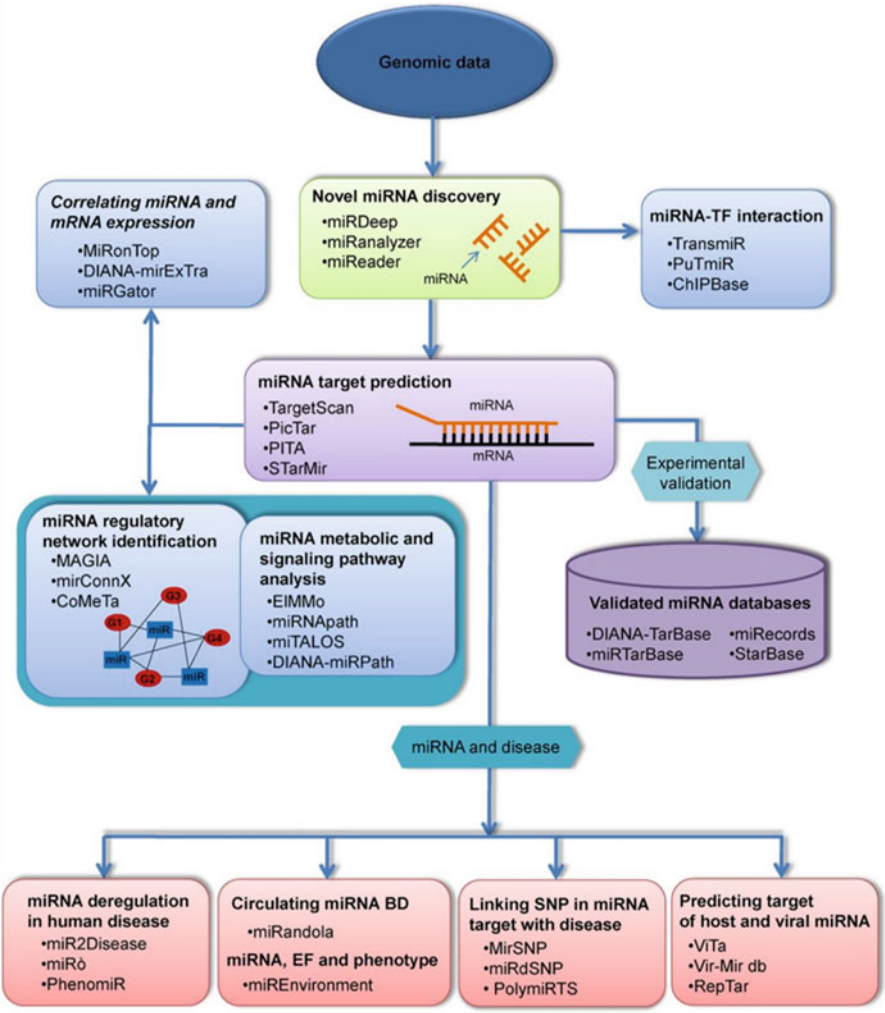


Fig. 3 Currently available bioinformatic tools classified based on principal type of data output. Illustrative examples of all categories are shown. (Reproduced with permission Akhtar et al. (2016). Copyright © 2015, the authors. Published under CC-BY 4.0 license)

Ultimately, experimental verification is an indispensable step to fully establish the miRNA::mRNA interaction encountered by computational methods. Regarding the database sites, miRBase currently serves as the reference for the miRNAs indexed in a number of species. miRBase encompasses a vast record of mature miRNA sequences identified in mouse and human and a much lower count in most other species including zebrafish, the increasingly used model for developmental research. Although this disparity may relate to lower genome content in the less documented species, it must also be interpreted as an indicator of differences in

gene annotation stringency (Desvignes et al. 2015) and sequencing efforts in the research carried out to date.

2.2 microRNA Isolation and Quality Check

miRNA isolation, also referred to as "extraction," is commonly the initial experimental step when studying expression level of any miRNA(s) of interest. Previously, the separation of nucleic acids from other tissue components was developed to yield RNA strands longer than 200 nt from any given sample (Lin et al. 2010). This separation could be obtained using one of the following techniques: guanidine isothiocyanate in β-mercaptoethanol followed by silica binding or phenol-chloroform phase exchange. To allow for the isolation of smaller RNA strands, including miRNAs, the combination of phenol-chloroform phase exchange and purification by silica binding in high polarity and the presence of nuclease-inhibiting guanidinium salts was introduced (Reddy and Gilman 2001; Farrell 2006). This method is currently supplied by several companies in the form of kits (Table 2) and yields total RNA above 10 nt with a high purity and integrity, including ribosomal RNAs as well as miRNAs and short housekeeping RNAs, i.e., the snoRNA family. With this recommended optimization, a subsequent enrichment procedure for small RNAs is generally not necessary, and the starting quantity of sample needed for a satisfactory yield remains small ($<1 \times 10^{5-6}$ cells). Ultimately, the effectiveness of the miRNA isolation process varies depending on the kind of sample and how it was collected and stored until

Table 2 Main commercially available platforms for miRNA research, with their respective suppliers

miRNA isolation (phenol + kit)	Array systems	PCR systems	Modulation	Reporter (target validation)
PureZOL + Aurum (Bio-Rad)	GeneChip miRNA (4.0) array (Thermo Fisher)	IScript + MystiCq (Bio-Rad)	miRCURY LNA (Qiagen)	LightSwitch™ 3′ UTR (SwitchGear Genomics)
QIAzol + miRNeasy (Qiagen)	miScript array (Qiagen)	miScript + SYBR Green (Qiagen)	MiRIDIAN/ shMIMIC lentiv. (Dharmacon/GE Healthcare LifeSciences)	pMIR-GLO™ (Promega)
TRIzol + PureLink (Thermo Fisher)	TaqMan A +B cards (Applied Biosystems)	TaqMan assays and TaqMan advanced assays (Applied Biosystems/Thermo Fisher)	miRVana Ambion (Thermo Fisher)	pMIR-REPORT™ (Thermo Fisher)
	TrueQuant SmallRNA Seq kit (GenXPro)			

the time of extraction; addition of a stabilizing agent like RNAlater® and performing mechanical digestion of the sample are technical steps that the researchers can consider to optimize this process in their specific work.

Once the miRNA isolation is completed, it is important to verify the quantity and quality of product obtained; spectrophotometry via NanoDrop is routinely used to do so. This technique uses just 1–2 μl of sample and performs an absorbance sweep between 200 and 300 nm; from the readout it is important to note the absorbance ratios at 260/280 nm and 260/230 nm, which should lay ~2.0 to indicate good RNA quality (Bernardo et al. 2012). The NanoDrop measurement can also inform genomic contamination when blanked with the same diluent (RNAse-free water) used for the isolation and observing the result of the DNA reading setting. Moreover, modern tools can also provide an RNA integrity number, with 8–10 indicating high RNA quality. However, more advance microfluidics platforms have also been developed – like the Agilent 2100 Bioanalyzer – to generate electropherograms of the peaks corresponding to the ribosomal RNA levels (5S, 18S, and 28S; Fleige et al. 2006). These systems perform an automated electrophoretic sequencing and render the ratios of those ribosomal species and their predicted bands upon northern blotting. This level of detail is of critical importance to proceed further with those samples onto microarray or deep sequencing experiments.

2.3 microRNA Profiling and Detection

Precision is the key for the utility of any miRNA profiling/detection technique, where identifying a particular miRNA and reporting its presence hold multiple challenges (Ozsolak and Milos 2011). These include the distinction between the precursors (pri-/pre-miRs) and mature forms of the given miRNA or the distinction among miRNA family members that present just one different nucleotide. The currently available techniques to achieve this task can be divided in high-throughput, i.e., microarray and deep sequencing, or low-throughput, like northern blotting, quantitative real-time polymerase chain reaction and in situ hybridization (Git et al. 2010).

High-throughput: These techniques are carried out in the early stages of exploratory miRNA research and provide large and highly valuable amounts of information that can serve as the foundation for multiple individual projects to follow (Kozarewa et al. 2009). *Microarrays* display probes matching a set of previously identified species-specific target miRNAs, thus serving to simultaneously test the level of expression of numerous miRNAs. This technique is performed seeking relative changes between conditions, such as healthy in comparison to diseased and treated compared with untreated. The limitation is the need for known sequence information to predesign the probes, although some companies have developed probes based on proprietary algorithms to potentially match unknown miRNAs (Bernardo et al. 2012). In contrast, *deep RNA sequencing* (RNAseq) does not rely on the input of publicly available sequence information for the predesign of the system. Instead, the sequencing platforms support the discovery of novel small RNA molecules, not limited to miRNAs, as well as longer RNA species like mRNA or

lncRNA. Additionally, this technique can also render information on the absolute expression level, or absolute abundance, eliminating the need to compare with a set reference. Although the capacity of the first platforms was focused on the identification of long DNA and RNA sequences, platforms such as the *Roche 454*, *Illumina*, and *SoLiD* currently have a much improved power to detect shorter strands. To date, the main factors limiting the use of RNAseq are the cost involved and the development of better computational tools to facilitate the storage, analysis, and interpretation of the complex and large datasets that this technique generates. Further developments in the systems biology area will permit the visualization of distinct datasets and facilitate the identification of novel interactions; this progress will be the key to enhancing uptake of high-throughput methods in tissue engineering. With new benchtop tools like Ion *Torrent* and *Miseq* beginning to emerge, together with the online software packages *Partek-RNAseq* (commercial), *miRanalyzer*, and *mirTools* (free), the accessibility of RNAseq seems likely to extend to many more TE researchers in the coming years.

Once the findings from the high-throughput techniques are obtained, the next step in the workflow is to verify these using some of the low-throughput options which retain better power to elude both false-positive and false-negative results (van Rooij 2011).

Low-throughput: *Northern blotting* is a classical semiquantitative technique based on electrophoretic properties and applicable to miRNA research, which uses polyacrylamide gels and nitrocellulose membranes. Although it requires big quantities of starting sample and arduous work, its advantages include robustness and resolution capacity to distinguish between mature miRNAs and their precursors. The sensitivity of this technique can be enhanced incorporating LNA technology and/or radioactive labeling in the design of the oligonucleotide probes (Várallyay et al. 2008). A more widely employed method to validate high-throughput results is *quantitative real-time PCR (qRT-PCR)*, with two variants in the reverse transcription (RT) reaction: universal or single detection of specific miRNAs with stem-loop primers (Varkonyi-Gasic and Hellens 2011). At the qPCR stage, the universal approach uses SYBR Green dye and both a linear miRNA-specific primer and a universal qPCR primer (Benes and Castoldi 2010). On the contrary, the single detection system is based on TaqMan® assays and, in addition to the dye label, always applies miRNA-specific primers (forward and reverse). Both options hold their pros and cons: single assays have constant pre-validated reaction efficiencies and sufficient specificity to differentiate mature from precursor miRNAs, but the separate RT reactions required make it costly and time-consuming; the universal method results in much more time and cost-effectiveness to study broader miRNA sets using substantially less starting amount of sample, but in exchange their discrimination between precursor and mature miRNAs is limited. There are three main considerations to ensure quality of the miR-PCR analysis: First, efficiency rate needs to be kept at 90–110% by adjusting a standard curve of miRNA, for example, with tenfold dilutions, to one-log amplifications every 3–3.5 cycles, especially in SYBR Green-based assays. Next, a melting curve must be added to ensure that the amplicon generated is of the right length. Finally, the selection of the reference gene for normalization must be standardized for each experimental model to ensure its sufficient and unaltered expression across the tested conditions. Frequently a

snoRNA or the mean of several snoRNAs can serve as the reference value to calculate the relative miRNA expression; however other housekeeping RNAs like the ribosomal 18S may also offer a stern reference. Another technique known as in situ *hybridization (ISH)* has been developed in support of the robust quantitative miR qRT-PCR results, which visually informs regarding the localization and spatiotemporal expression patterns of miRNAs (Obernosterer et al. 2007). ISH is recommended for the detection of miRNAs known to be highly expressed but can be troublesome when targeting rare miRNAs. As previously seen with northern blotting, LNA-detection probes appear to be a promising option to improve miRNA ISH (Silahtaroglu et al. 2007). This technique can furnish our understanding of many miRNA functions and can be optimized for both frozen- and paraffin-embedded tissues, by tuning the fixation procedures and introducing amplifier chemicals for the tyramide signals, as seen in the work by Silahtaroglu et al. (2007) and Pena et al. (2009).

2.4 Studies of Target Validation and Biological Effects

After a microRNA and its expression profile in a given physiological or pathological state has been elucidated, the next target typically focusses on its biological impact, which starts by identifying the direct target of such miRNA (Ørom and Lund 2010). Bioinformatic prediction algorithms discussed earlier in this chapter are very useful drivers at this stage of the process, but the immediate following step is to experimentally validate the existence of such predicted interactions (Bernardo et al. 2012). In order to do so, the most widely extended approach is to carry out luciferase reporter assays. In these assays, the 3′-UTR region of the putative mRNA target of the miRNA of interest is cloned downstream of a luciferase reporter plasmid vector (Fig. 4 and Table 2). Upon confirming the successful construction and expression of this reporter vector, the cells of interest are exposed to the 3′-UTR reporter plasmid using a conventional transfection method. Additional groups will receive a co-transfection with a synthetic miRNA mimic/inhibitor; see Sect. 3.1 for details on how these work. Further controls including the vector with a mutation in the 3′-UTR region and a non-targeting miRNA (or *scrambled*) shall be included in the experiment (Fig. 4). Following a short incubation time, samples are to be collected and analyzed for luciferase activity. Substantial changes in luciferase activity across the groups will indicate that a direct interaction exists with the miRNA mimic or inhibitor, which is capable of altering the expression of a functional protein.

Typically, a positive result would show that, compared with the cells that only received the luciferase reporter vector, the cells co-treated with the miR-mimic present lower luciferase activity, whereas the cells that co-treated with the miR-inhibitor present higher luciferase activity. The scrambled treatment should not alter the reporter luciferase activity vs. the vector only cells and neither should the miR-mimic when co-transfected with the mutated 3′-UTR reporter.

An alternative to these reporter assays is the comparative transfection with miR-mimics and inhibitors followed by the assessment of putative targets at the

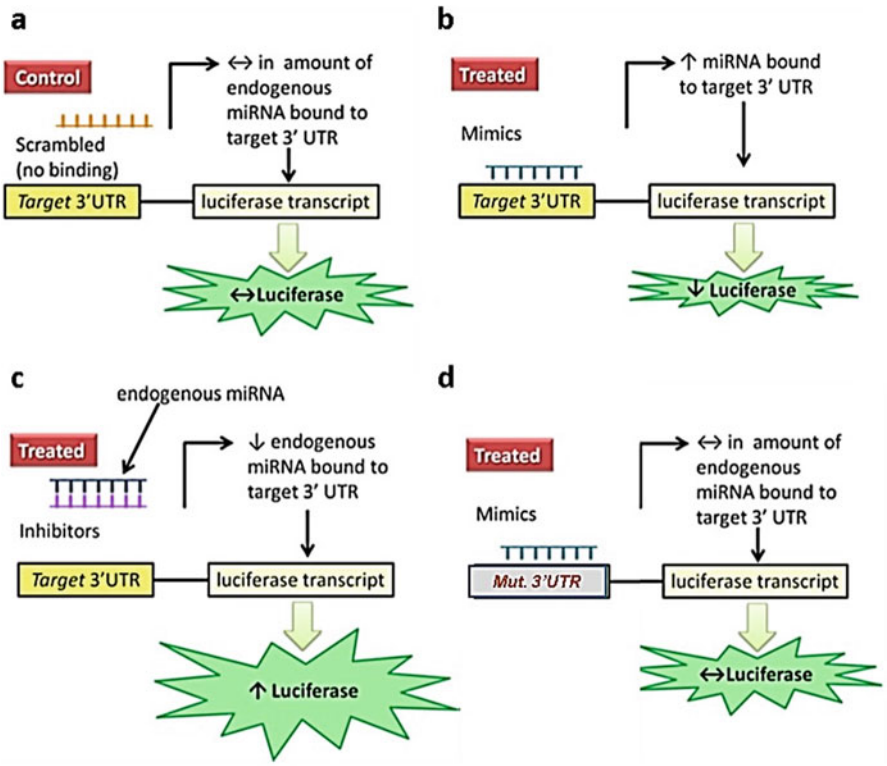


Fig. 4 Diagram of luciferase reporter assays for miRNA::mRNA interaction validation. (Adapted with permission Bernardo et al. (2012); Copyright © 2012; Elsevier B.V. All rights reserved). Cells transfected with (**a**) scrambled control, (**b**–**c**) the miR-mimic or the inhibitor, in addition of the reporter construct or the (**d**) mutated reporter construct, will result in different levels of luciferase expression, whose activity is readily quantified with commercially available kits

mRNA level – by qPCR – and/or at the protein level, via western blotting or immunostaining. However, detecting changes in the levels of the putative target following this approach would not rule out the possibility of an indirect interaction with the miRNA of interest. Furthermore, the effect of successfully transfected miR-mimics and inhibitors on the expression of its direct mRNA target tends to be of moderate amplitude, i.e., 0.5-fold reduction or 1.5-fold increase. This complicates the clear detection of the sought interaction using said PCR and immunostaining techniques, sometimes prompting researchers to explore methodologies like pull-down assays, proteomics, and even transcriptomics. For further details on the molecular techniques fit to evaluate the presence and levels of the putative target, which are not specific to miRNA research but general to protein studies, we direct the reader to other available reviews (van Rooij 2011; Ørom and Lund 2010). Ultimately, a confirmation of the validated direct interaction between a particular miRNA and its mRNA target of interest for a specific application is essential to

continue the evaluation of the biological implications of that given miRNA. It is important to note that abundant information on validated miRNA::mRNA interactions can be found from the literature or compiled in bioinformatic databases. Thus, a project pursuing the therapeutic application of a certain miRNA can frequently be designed based on such externally reported information; this is indeed an extended practice in the application of miRNA therapeutics to tissue engineering. However, consideration must be given that validation of a miRNA::mRNA interaction in a particular cell type, animal model, or species does not ensure consistency of that interaction in a different setting. It is recommended hence to perform preliminary studies of species homology at the bioinformatics level and also to determine the basal expression of the miRNA and its target experimentally, before aiming to assess a potential therapeutic effect.

Focusing on the exploration of the biological or therapeutic effects of miRNAs, multiple options are worthy of consideration, ranging from in vitro tests to preclinical evaluation using animal models. As a common denominator of this phase of the miRNA research, the effect of miRNA overexpression or inhibition is evaluated for phenotypic – and sometimes also genotypic – changes. The key elements of resolution when planning in vitro or in vivo miRNA experiments should be:

1. Determination of the timing and dose of mimic/inhibitor that is effective and nontoxic – for cells and tissues – delivered to the specific model
2. Establishment of the time-course of treatment and analysis points, testing guided by literature reports
3. Comparative evaluation of the effect, i.e., across two cell types or culture conditions, to obtain a broader insight in the amplitude and impact of the biological response
4. Confirmation of efficient target silencing to ensure system reproducibility
5. Assessment of downstream targets and markers standardized for the application of interest
6. Evaluation of biodistribution and adverse effects of the optimized treatment to main organs like the liver and kidney (Bernardo et al. 2012)

The next sections of this chapter will provide a detailed review of the current knowledge regarding the role of miRNAs in tissue repair and the routes by which those roles have been harnessed to date to provide improved therapeutic options in the various subfields of tissue engineering and regenerative medicine.

3 miRNA Modulation as a Therapeutic for Cell and Tissue Engineering

The emerging understanding of miRNA function and regulation has sparked their exploration in the development of more advanced therapies for tissue engineering applications (Hu et al. 2010; Jensen et al. 2010; Haussecker 2014). The competitive advantage of miRNAs over other gene therapy variants is multifold, but

perhaps the main point resides in the ability of a single miRNA to imperfectly bind to a multitude of targets, resulting in a multi-targeting effect capable of modulating complex signaling pathways (van Rooij 2011; van Rooij et al. 2012; Bader et al. 2010). Such an effect potentially incurs a robust and enhanced biological response (Beavers et al. 2014). Other additional benefits of miRNAs are their small molecular size and cytoplasmic activity, which can simplify delivery methods as they do not depend on access to the cell nucleus or transcription machinery. Furthermore, the possibility of inhibiting or blocking the function of miRNAs to achieve the upregulation of their direct target provides a tool for the bidirectional control of gene and protein expression. Consequently, the landscape of miRNA-related patents denotes fast-paced progress with positive projections for commercialization, with over 500 filed US patents related to miRNA-based therapies as of 2015 (Fig. 5; Christopher et al. 2016). This includes inventions in areas of wound healing, bone, muscular, ocular, respiratory, and cardiovascular tissues, as well as inflammatory diseases, approximately accounting for nearly one fifth (20%) of the total filings, which collectively underlines the high impact of miRNA therapies in tissue engineering (Monaghan and Pandit 2011; Chew 2015). Taken together with its speedy progression to enter clinical trials, this suggests that miRNA therapy holds great promise as an effective protein modulating alternative to the delivery of high doses of protein (Takeda 2009; Vo et al. 2012).

The first candidates entering clinical trials were the liver-targeted miravirsen and MRX34 (Janssen et al. 2013; ClinicalTrials.gov 2013), because this organ retains the highest concentrations of unmodified miRNAs upon systemic administration. Further progress currently includes Phase I/IIa trials for nonalcoholic fatty liver disease and type 2 diabetes sponsored by Regulus Therapeutics or Phase I trials for mesothelioma and scleroderma. Moreover, many miRNAs are in the R&D pipeline as tissue repair treatments for diseases of the circulatory system including miR-92a, miR-33, miR-15, and miR-208 or diseases caused by uncontrolled fibrosis or inflammation such as miR-21 and miR-155.

3.1 Types of miRNA Therapeutics

As outlined earlier in this chapter, synthetic molecules exist to modulate – i.e., mimic or inhibitor – the natural activity of miRNAs in a cell type of interest and thus render a therapeutically engineered cell phenotype with a physiological miRNA profile (van Rooij et al. 2012; Fig. 6). The design of synthetic miRNA variants includes sequence editing and chemical modifications to tailor the affinity of binding to the target or provide increased cellular uptake and stability against degrading enzymes. These include the locked nucleic acid (LNA) technology developed by Exiqon (Braasch and Corey 2001; Petersen and Wengel 2003) or the phosphate backbone functionalization which creates hybrid peptide nucleic acids (PNA) (Fabani and Gait 2008; Table 3). These modifications can be applied simultaneously within a single nt but not in every unit of the oligo chains; therefore perfecting the balance between unmodified and multi-modified units is a task

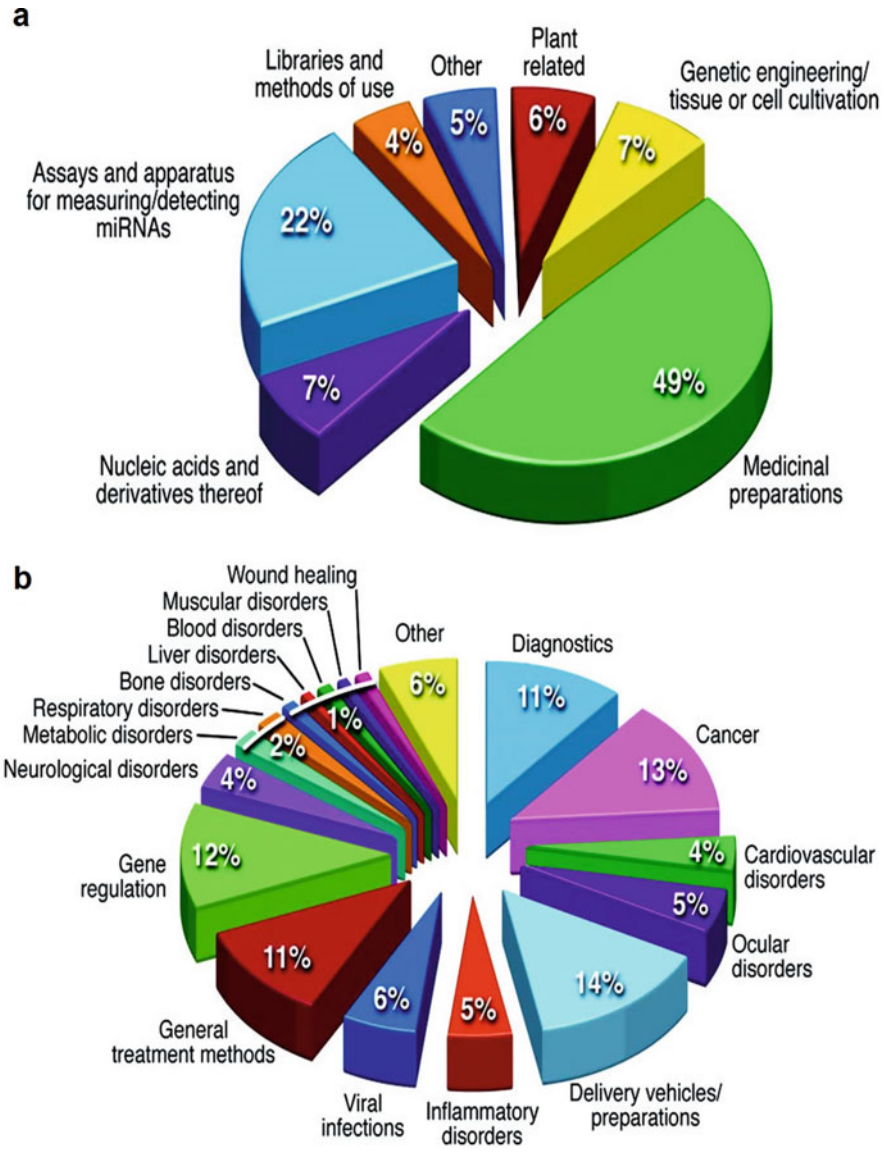


Fig. 5 miRNA-related distribution of US patents. (Reproduced with permission van Rooij et al. (2012). Copyright © 2012, American Heart Association, Inc). (**a**) The distribution of different technological fields that incorporate applications of miRNA, as determined by International Patent Classification codes. (**b**) Distribution of filed patents related to miRNA-based medicinal preparations divided by particular indications

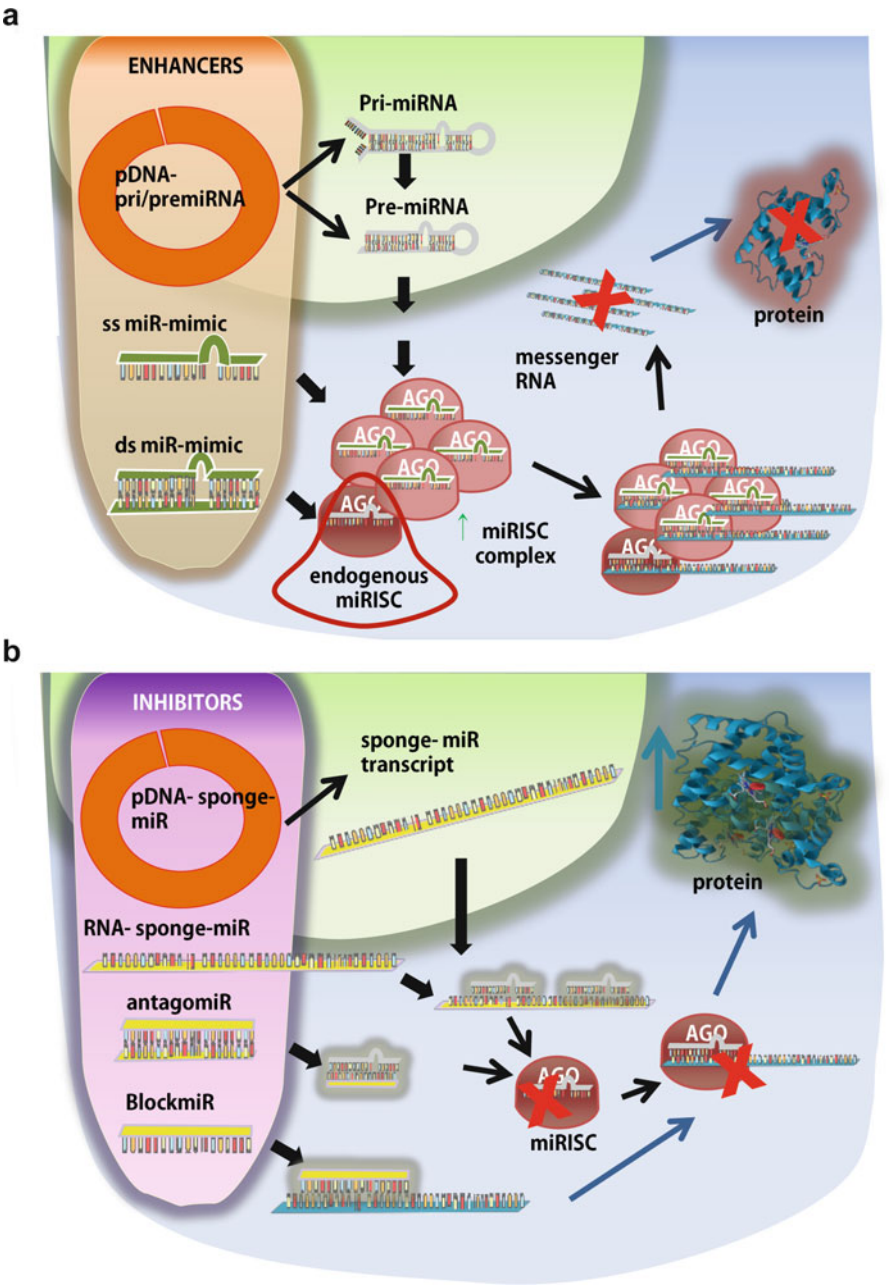


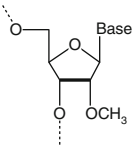
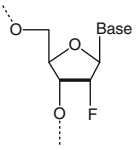
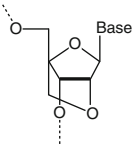
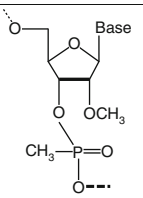
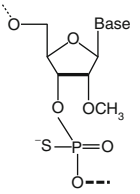
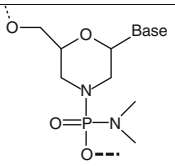
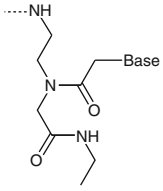
Fig. 6 Types of miRNA modulators. (Reproduced with permission from (Curtin et al. 2017). Copyright © 2017, John Wiley and Sons). (**a**) miR-mimicking by pDNA encoding for miRNA precursors, single- or double-stranded oligonucleotides serve to enhance the formation of miRISC

which garners intensive work. This is especially so with single-stranded miR-mimics because their guide strand must be recognized as identical to the miRNA of interest to form the miRISC complex (van Rooij and Kauppinen 2014). However, double-stranded miR-mimics can also be used; these can feature a more modified chemistry in the passenger strand, rendering potency improvements of above 1000-fold in comparison to the single-stranded molecules. An alternative strategy to enhance the level or activity of endogenous miRNAs is the application of plasmid (p)DNA vectors coding for precursors of the miRNA, instead of direct delivery of the mature miRNA form. This strategy remains cost-effective as bacterial cultures allow continuous propagation of the vector; however, it presents higher risks of overloading the enzymatic activity of endogenous miRNA biogenesis (Bonadio et al. 1999).

Focusing on the options for miRNA inhibition, we can distinguish three categories which are antagomiRs, miR-sponges, and small chemical inhibitors (Fig. 6b). *AntagomiRs*, also named "antimiRs" or "AMOs," are short antisense oligonucleotides which have high binding affinity for their target miRNA, owing to their modified chemistry; this pairing impedes the interaction with the mRNA, whose levels and translation into protein are effectively increased (Bernardo et al. 2012). Hence, antagomiRs have the capacity to modulate the various mRNA targets of their inhibited miRNA and are the first effective miRNA inhibitors preclinically that have progressed to clinical trials (Krutzfeldt et al. 2005). *BlockmiRs* are a subtype of short antisense inhibitors able to selectively engage with a single target mRNA in the region that would bind the desired miRNA but that support protein translation instead of entry in the miRISC. *miR-sponges* are long and single strands that function via competitive substitution of the mRNA in its interaction with the corresponding miRNA, ultimately confiscating away multiple miRNA copies at a time and thus preventing their activity (Ebert et al. 2007; Ebert and Sharp 2010). miR-sponges are usually constructs of pDNA vectors, like miRNA eraser, miRNA mower, tough decoy (TuD), and LidNA, and are preferred as a stable genetic modification to treat chronic diseases. Finally, *small chemical inhibitors* like dihydropteridinone ATP analogues, diazobenzenes, and helix-threading peptides act by impeding miRNA biogenesis unspecifically (Sakurai et al. 2008). Although they are valuable in developmental studies and for their good pharmacokinetics, they present an enhanced risk of side effects in comparison to the other types of inhibitors (Deiters 2010; Zhang et al. 2010).

Fig. 6 (continued) complex alongside endogenous miRISC, ultimately leading to decreased levels of messenger (m)RNA or protein of the target present in the cell. (**b**) miRNA inhibition by pDNA/RNA sponges or antagomiRs (antimiRs) impedes the entry of the mature miRNA to form miRISC and abrogates downstream interaction with the target mRNA. "BlockmiRs" conceal the binding sites of mRNA directly preventing its entry in miRISC. The three approaches act to ultimately enhance mRNA and protein levels of the target in the cell

Table 3 Effects of chemical design of miRNA therapeutics. (Reproduced with permission from Curtin et al. (2017). Copyright © 2017, John Wiley and Sons)

Types of modification	Chemical structure	Function	Properties conferred
Ribose methylation: 2'O-Me		• Enhances hydrophobicity • Increases duplex melting temperature	↑ Cellular uptake
Ribose fluoridation: 2'F		• Enhances hydrophobicity • Increases duplex melting temperature	↑ Cellular uptake ↑ Stability
Conformational ribose locking: LNA		• Ribose ring structurally locked in C3'-endo conformation • Enhances hydrophobicity • Improved target-binding affinity	↑ Cellular uptake ↑ Bioactivity
Phosphate backbone methylation: P-Me		• Improved enzymatic resistance • Enhances hydrophobicity • Increases duplex melting temperature	↑ Cellular uptake
Phosphate backbone thyolation: PS		• Introduces phosphorothioate linkages • Improved enzymatic resistance • Enhanced binding to plasma proteins • Improved pharmacokinetics	↑ Stability ↑ Bio-distribution time, slowed excretion
Morpholino-ribose substitution: PMO		• Introduces phosphorodiamidate linkages • Removes negative charge • Improved target-binding affinity	↑ Cellular uptake ↑ Bioactivity ↑ Stability
Peptide-phosphate backbone substitution: PNA		• N-(2-aminoethyl)-glycine units substitute the phosphaste backbone • Removes negative charge • Improved enzymatic resistance	↑ Cellular uptake ↑ Stability

3.2 miRNA Delivery Methods

The delivery of miRNAs presents multiple technical challenges due to their negative charge, rigidity, and low charge density (Pan et al. 2012a; Lee et al. 2014), as well as their susceptibility to enzymatic degradation and poor intracellular cytosolic delivery, owing to the lysosomal digestion process. These difficulties explain the limited success obtained to date with the delivery of naked miRNAs and the need for delivery methods that can function safely and efficiently. As such, the field of miRNA delivery accounts for nearly a third of the total share of the miRNA-related US patents, representing a bigger portion than the ~20% of the combined tissue engineering and regenerative medicine (TERM) areas mentioned at the beginning of this Sect. 3 (Monaghan and Pandit 2011; Chew 2015). miRNA delivery systems specifically developed for tissue engineering purposes often combine vectors and biomaterial scaffold platforms to spatiotemporally sustain modulation of miRNA and target. This subsection describes the main properties and highlights applications of both vectors and scaffolds, which are roughly transferable across systems developed to deliver pDNA, siRNA, or miRNA. A series of prerequisites are generally asked of a successful delivery system – summarized in Table 4. The continuous efforts in this area no longer aim to develop a single ideal system fitting all applications; instead, the goal is to satisfy as many of these prerequisites as possible while using fine-tuned platforms for each particular application.

3.2.1 miRNA Delivery Vectors

The principal role of the delivery vectors is to improve cellular uptake and prevent lysosomal degradation, and they are broadly divided into viral and non-viral methods (Ilina et al. 2012; Midoux et al. 2009). Generally, *non-viral vectors* present adequate properties with regard to fabrication, safety, and stability but have modest functionality (Santos et al. 2011). Non-viral vectors are associated with temporal and variable transfection efficiencies per cell type (Midoux et al. 2009; Santos et al. 2011; Pack et al. 2005). However, the temporal effect is beneficial in tissue

Table 4 Prerequisites of miRNA delivery vectors to progress toward clinical applications

Fabrication	Safety	Stability	Functionality
Ease of fabrication	Safe administration route	Robustness/stability (chemical)	Internalization
Inexpensive	Nonpathogenic	In physiologic fluids, including serum	Endolysosomal escape
Facile purification	Nontoxic	For storage and administration	Entry in nondividing cells
Targetability (to specific cells/tissue)	Non-immunogenic	Protection of cargo	Nuclear transport (generally not RNAi)
		Packaging sufficient amount of cargo	Efficient unpackaging/packaging

engineering to allow the termination of tissue repair without undesired tissue overgrowth. Although non-viral physical transfection methods like electroporation or microinjection offer consistently high efficiencies, they are relatively invasive techniques and complicated to adapt to off-the-shelf tissue engineering therapeutics. Meanwhile, the opposite occurs with chemical vectors: a plethora of highly adaptable materials and nano-sized systems that allow noninvasive miRNA delivery by forming complexes via electrostatic interaction, charge adsorption, or physical encapsulation. These include natural and synthetic polymers, generally with cationic properties. Among the cationic polymers, polyamines are common structural features that provide an efficient path to escape lysosomal degradation. This path, known as the "proton sponge" effect, attracts anions to disrupt the endolysosomal compartment before the degradation can start in the mature lysosome, thereby allowing the miRNA cargo to be released into the cytoplasm (Behr 1997). To optimize this effect for each cell type, the ideal proportion of polyamines ("N") to phosphate groups ("P") present in the cargo, i.e., the N/P ratio, must be determined. The principal synthetic cationic polymers explored for miRNA delivery are derivatives of polyethyleneimine (PEI) (Zhang et al. 2013; McKiernan et al. 2013; Schade et al. 2013, 2014), poly(β-amino esters) (PβAE), methacrylates, dendrimers (Raftery et al. 2016), cyclodextrins (Fitzgerald et al. 2015, 2016), and cell-penetrating peptides (CPP) (Guo et al. 2016; Sathy et al. 2017); examples and their main limitations are described below.

Beyond commercially available transfection agents like ExGen500 and jetPEI™, PEI derivatives have shown high miRNA transfection efficiencies both within poly (lactic-co-glycolic acid) (PLGA) microspheres embedded in poly(L-lactic acid) (PLLA) scaffolds (Zhang et al. 2016a) and alginate-based hydrogels (Liu et al. 2016). The main drawback of PEI derivatives is their tendency for exacerbated proton sponge effects that generate notable cell damage and thus compromise the clinical applicability (Ghosh et al. 2012). Contrastingly, PβAEs display low cytotoxicity while keeping high transfection efficiencies (60–70%) in various cell types including hMSCs and human adipose-derived stem cells (ADSCs) (Vuorimaa et al. 2011; Yang et al. 2009; Guk et al. 2013; Yin et al. 2011; Jere et al. 2008; Remaut et al. 2010) and also allow kinetically controlled biodegradation for temporal delivery of their cargo (Gupta et al. 2015). Recent innovations in methacrylate-based miRNA/siRNA delivery systems, dendrimers, cyclodextrins, and CPPs offer the main overall advantage of chemical versatility. These provide precise physicochemical tuning and thus control of the pharmacokinetic profile, hence holding great potential for incorporation into scaffold-based platforms as miRNA delivery vectors. In particular, a dimethylaminoethyl methacrylate copolymer formulated as a dendrimer with polylactic acid (PLA) (Wang et al. 2017a; Nelson et al. 2014) demonstrated cargo protection, adequate cytosolic release, and significant gene knockdown when delivering miR-21 as a treatment for glioma (Qian et al. 2014). Additionally, advanced polyamidoamine (PAMAM) dendrimers have also demonstrated highly efficient macropinocytosis-mediated delivery of a miR-205 mimic or an antagomiR-221 within a hydrogel to treat breast cancer (Conde et al. 2016; Haensler and Szoka 1993).

Interestingly, the non-viral vectors above described remain underutilized compared to the extensively researched *lipid-based cationic vectors*. FuGENE, GenePORTER, TransFast, DOTAP, Lipofectamine® 2000, and Lipofectamine RNAiMAX are but a few examples of widely commercialized lipid transfection agents (Wu et al. 2013; Eskildsen et al. 2011; Wang et al. 2015; Hoseinzadeh et al. 2016; Chen et al. 2011a; Li et al. 2013). These lipid vectors are often presented as liposomes, spherical bilayers of polyamines, and cholesterol that easily fuse with cell membranes to aid the uptake of their miRNA cargo. Typically, the cationic lipids interact electrostatically with the miRNAs resulting in a net positive charge, while core encapsulation of the cargo occurs more frequently with liposomes of neutral charge. Liposomes offer a vast versatility, a distinctively high loading capacity, and a renowned high efficiency, but their main clinical drawback is their immunogenicity and toxic-detergent effect on cell membranes, generating intracellular vacuoles (Li et al. 2014). With intensive efforts on developing vectors that lack these cytotoxicity issues, materials of a natural base like chitosan and inorganic nanoparticles have garnered the excitement of tissue engineering researchers (Ghosh et al. 2012). *Chitosan* is a crustacean-derived polysaccharide with commendable biodegradability and biocompatibility and was introduced as a gene delivery vector approximately two decades ago (MacLaughlin et al. 1998). Altering its polymerization, acetylation degree, and/or chain architecture allows the optimization for miRNA delivery (McKiernan et al. 2013; Malmo et al. 2012), with highly depolymerized chitosan showing optimized miR-145 delivery in adenocarcinoma studies with approximately 50% target silencing (Santos-Carballal et al. 2015).

Nanoparticles (NP) assembled from inorganic materials like gold (Au), silver (Ag), iron (Fe), and calcium phosphates (CaP) also hold interesting properties. *Au-based NPs* are amphiphilic vectors with demonstrated ability to deliver miRNA reporters in cancer applications like ovarian cancer or neuroblastoma (Ghosh et al. 2012). *Silver- and iron-based systems* include additional physical triggers such as light or magnetism to switch on and off the miRNA delivery; this offers on-demand spatiotemporal control over miRNA delivery with high precision in surface body areas. Relevantly, an 80% uptake efficiency range was reported for photocleavable Ag NPs delivering miR-148b mimic to hADSCs (Qureshi et al. 2013) and paramagnetic Fe_3O_4 NPs delivering miR-335 to hMSCs to treat cardiovascular diseases (Schade et al. 2013). Finally in this group are the *CaPs*, biodegradable and highly biocompatible ceramics naturally found in bone (Pedraza et al. 2008; Cunniffe et al. 2016) which can be fabricated easily and cost-effectively (Bose and Tarafder 2012; Wu et al. 2008). CaP-aided transfection was first reported in 1973 (Graham and van der Eb 1973), and it facilitates cellular uptake by dynamin/clathrin-endocytosis after precipitating at the cell surface (Ilina et al. 2012). CaPs feature low pDNA delivery efficiency and instability in solution (Li et al. 2010) but have been surface-modified with PEG, chitosan, hyaluronic acid, and 3,4-hydroxyphenylalanine-dopa for improved efficiency, specificity, and loading capacity (Lee et al. 2013, 2014; Wu et al. 2008; Zhang et al. 2009; Giger et al. 2013). Non-aggregating CaPs with hydroxyapatite stoichiometry (HA; Cunniffe et al. 2010) proved superior for hMSC transfection when compared to a commercial CaP kit (Curtin et al. 2012),

with 90% functional efficiency of reporter miR-mimics and antagomiRs (Mencia Castano et al. 2015); a scaffold system incorporating these has demonstrated potential as a new bone repair therapeutic (Mencia Castano et al. 2016, 2018).

To conclude this section on miRNA delivery, *viral vectors* are worthy of discussion, as they lead gene therapy development with the recently approved Zalmoxis and Strimvelis, by MolMed and GlaxoSmithKline, respectively ((Agency), E.E.M 2016a, b). As nature's transfection agents, viruses have characteristically high transfection efficiencies with little variability. Viral miRNA therapy for tissue engineering might be of interest to permanently correct endogenous levels of a miRNA in chronic degenerative diseases like OA. It is worth highlighting that viral miRNA delivery has proven reduced dose-dependent toxicities versus shRNA and siRNA, with improved silencing efficiency (Boudreau et al. 2009). The principal drawback of viral miRNA delivery is insertional mutagenesis, although the risks of fatal effects are much reduced in lentiviruses versus retroviruses (Laufs et al. 2006; Montini et al. 2009). This has possibly swayed the greater use of lentiviral-based systems combined with biomaterial scaffolds (Deng et al. 2013a, 2014a; Xie et al. 2016), although adenoviruses and adeno-associated viruses are widely used in in vitro miRNA transfection studies (Mowa et al. 2010) which may be due to their reduced size and non-pathogenicity for humans (Liu and Berkhout 1809). More recently, vast attention has been directed to baculoviruses, which also lack pathogenicity in humans and present reduced risks by not replicating or integrating in mammalian cells (Chen et al. 2011b; Liao et al. 2014a). Clinical translation of viral miRNA approaches remains hindered also by difficulties in up-scaling the complex production and the high costs associated with this (Gordeladze et al. 2010). Viruslike particles, consisting of noninfectious recombinant variants of virus capsids that maintain the original cell tropism and trafficking capacities (Pan et al. 2012a, b), may offer a valuable tool to accelerate clinical progress.

3.2.2 Application of Biomaterial Scaffolds to miRNA Delivery

The concept of using biomaterial scaffolds, originally designed to support tissue growth, as therapeutic delivery devices is now widely established among tissue engineering researchers (Lee et al. 2011). Evolving from the "gene-activated matrix" idea which surfaced at the end of the 1990s (Bonadio et al. 1999; Fang et al. 1996), scaffold-based miRNA delivery is now growing (Chew 2015). This approach offers physical shielding from degrading agents and delays the clearance of miRNAs from the target site, enhancing the time frame of therapeutic effects within a localized area (Bonadio et al. 1999; O'Brien et al. 2007; Sriram et al. 2015; Nguyen et al. 2014). In this way, scaffold-based miRNA delivery may minimize the biodistribution to off-target tissues, representing an important advantage in terms of safety (Chew 2015). Importantly, the 3D microenvironment has been shown to alter the transfection efficiency of the vectors employed, in comparison to monolayer settings, which has been associated with changes in treatment exposure within the scaffold matrix. This highlights the need to perform extensive in vitro testing of the scaffolds once activated with the miRNA therapeutic. In this section we review the main properties required to successfully adapt the different materials and existing scaffold types as miRNA delivery platforms, while their therapeutic application is summarized in Table 5 and later detailed in Sect. 5

Table 5 Summary of miRNA therapeutic potential reports in tissue engineering using scaffold-based delivery. (Reproduced with permission Curtin et al. 2017. Copyright © 2017, John Wiley and Sons)

Scaffold type	Vector type	Therapeutic	Endpoint analysis result	In vivo model/cell type	Ref.	Appl.
Gelatin-coated PLGA scaffold disc	Baculovirus	ASCs, BMP2+ miR-148b mimic transduced	12 weeks: complete bridging: bone area = 94%, BV = 89%, BD = 95%	4 mm critical-sized calvarial defect in nude mice	Liao et al. (2014a)	Bone
Gelatin-coated PLGA scaffold disc	Baculovirus	ASCs, BMP2+ miR-148b mimic transduced	12 weeks: filling 83% defect area, 75% BV, bone density 89% of original	4 mm critical-sized calvarial bone defects in nude mice	Sung et al. (2013) and Mariner et al. (2012)	
Gelatin-spongostan scaffold	Baculovirus	OVX-BM-MSCs transduced with miR-214 or miR-140 sponges	Bone healing, bone density, trab. no, trab. thickness, and trab. space: further synergy by BMP2 co-expression	Osteoporotic rat femoral metaphysis defect model	Li et al. (2016a)	
Poly(glycerol sebacate) porous scaffold discs	Lentivirus	BM-MSCs, miR-31 inhibitor transduced	8 weeks postoperatively: significant improvement in healing vs. control groups (near full bridging)	8 mm calvarial rat defect (12-w.o. male Fischer 344)	Deng et al. (2014a)	
β-TCP scaffold	Lentivirus	ASCs or BM-MSCs, miR-31 inhibitor transduced	Bone regeneration in 8 weeks in rat model and in 16 weeks in canine, Runx2-Satb2 loop mechanism	5 mm rat calvarial defect, 10 mm canine orbital bone defect	Deng et al. (2013a, 2014b)	
Poly(sebacoyl diglyceride) (PSED) scaffold	Lentivirus	BM-MSCs, miR-135 mimic or inhibitor transduced	8 weeks: respectively enhanced or decreased newly formed bone and trabecular number	8 mm critical-sized rat cranial defects	Xie et al. (2016)	
PSED scaffold	Lentivirus	BM-MSCs transduced with miR-125b inhibitor	8 weeks: enhanced SMAD4, Runx2 and Osterix expression in newly formed bone	8 mm critical-sized rat cranial defects	Xie et al. (2017)	

Demineralized bone matrix	Lentivirus	BM-MSCs transduced with miR125b inhibitor	51% increased BMD, 79% increase in Tb.N; histologically enhanced new bone generation and bone maturity	Bilateral segmental femoral defects in athymic mice	Wang et al. (2017b)	
Nanofibrous PLLA scaffold	HP-PEI-PEG	26a mimic (cell-free in vivo)	Bone regeneration in 8 weeks in both healthy and osteoporotic mice	5 mm critical-sized mice calvarial bone defect	Zhang et al. (2016a)	
HyStem-HP™ and Hya/gelatin/ PEG hydrogels	siPORT NeoFx™	hBM-MSCs, miR-26a mimic transduced	Histomorphometry at 8 weeks (ectopic model): increased bone formation vs. control; micro-CT analysis at 12W (calvarial defect model) full bridging	3 mm and 5 mm mice calvarial defect (nude C57BL6J), mouse (m) or hBM-MSCs	Li et al. (2013)	
HA/TCP scaffold disc	Lipofectamine® 2000	Rat ASCs, miR-26a mimic transduced	Histomorphometry at 12 weeks: increased new bone forming area vs. control groups	3.5 mm diameter rat cortical tibial defect	Wang et al. (2015)	
Matrigel™ or PCL scaffold	PC-SilverNP	Pre-miR-148b-transduced ASCs	4 and 12 weeks: healing improved 32.53 ± 8.3%	4 mm critical-sized mouse calvarial defect	Qureshi et al. (2015)	
Porous collagen-nHA scaffold	nHA particles	GAPDH targeting miR-mimic, antagomiR-16	Silencing functionality of ~ 20% and 88.4%, respectively	hMSC, 2D and 3D delivery profile characterization	Mencia Castano et al. (2015)	Bone
Porous collagen-nHA scaffold	nHA particles	antagomiR-133a-3p	Enhanced Runx2 and osteocalcin expression, increased alkaline phosphatase activity and Ca^{2+} deposition	hMSC, 2D and 3D assessment of therapeutic osteogenesis	Mencia Castano et al. (2016)	
EXg 3D scaffold	Naked	miR-2861	miR-2861 and RunX2 overexpression	Human periodontal ligament (hPDL) SCs	Diomede et al. (2016)	

(continued)

Table 5 (continued)

Scaffold type	Vector type	Therapeutic	Endpoint analysis result	In vivo model/cell type	Ref.	Appl.
Atelocollagen	Naked	miR-222 inhibitor	8 weeks: positive radiographic, μCT and histological evaluation	Rat refractory fracture model	Yoshizuka et al. (2016)	
PEG-norbornene hydrogel	Naked-nucleofection	miR-148b mimic + miR-489 inhibitor	66% increase alkaline phosphatase activity and fourfold increase in Ca^{2+} deposition (day 10) over untreated control	hBM-MSC, 2D and 3D assessment of therapeutic osteogenesis s	Mariner et al. (2011)	
Alginate hydrogel +osteochondral biopsy	RNAiMAX	BM-MSCs transduced 2X with miR-221inhibitor	12 weeks: cartilage repair with absence of coll X	Subcutaneously implant in nude mice	Lolli et al. (2016)	Cartilage
Fibrin hydrogel	siPORT NeoFx™	antagomiR-133a and anatagomiR-696	Enhanced expression of PGC-1a, and contractile force	2 weeks myotubes culture	Cheng et al. (2016)	Muscle
PEG-PCL-gelatin electrospun fibers	REDV peptide-trimethyl chitosan-PEG	miR-126	8 weeks: in vitro downregulation of SPRED-1, improved endothelialization in vivo	Rabbit 1 cm left common carotid artery resection	Zhou et al. (2016)	Cardiovascular
Fibrinogen, Matrigel™ and thrombin hydrogel	DharmaFECT	miR-1+ miR-133+ miR-208+ miR-499	MMP-dependent mechanism of successful reprogramming	Direct reprogramming of fibroblasts into cardiomyocytes	Li et al. (2016b)	

4S-StarPEG collagen scaffold (gel)	Naked	miR-29b	4 weeks: reduced wound contraction, improved coll III/I ratio, higher MMP-8: TIMP-1 ratio	Full-thickness rat skin excisional wounds	Monaghan et al. (2014, 2018)	
Glycosan HyStem hydrogel			2 and 5 weeks following MI: myocardial function maintenance at	Mice ischemia reperfusion model		
Pluronic F127 gel + vein segments	Lentivirus	miR-26a mimics	4 weeks: reduced hyperplasia and inhibited SMCs proliferation	In rat vein neointimal hyperplasia model	Tan et al. (2017)	
Electrospun PCL fiber scaffold	TransIT-TKO	miR-219 and miR-338		OPC remyelination	Diao et al. (2015a)	Neurological
Sed scaffold	n/a	miR-20	Unraveling miR signature	NSCs self-renewal and differentiation control	Cui et al. (2014)	
Aminopropyl-silica nanofibers	n/a	Let-7			Mercado et al. (2016)	

In addition to the general principles for apt biomaterials such as ease of fabrication, biodegradability, and biocompatibility (O'Brien et al. 2007), ease of sterilization and long-term stability are key limiting requisites from the angle of clinical translation (Bucholz 2002). However, the most specific requisite for any miRNA delivery scaffold is the structural ability to retain the miRNAs and sufficiently expose them to the infiltrating cells, thereby mediating the localized miRNA release while not impeding cellular uptake. Thus, a balanced interaction strength must be met between scaffold and miRNA complexes (Zhang and Webster 2009). For this reason, highly porous scaffold architectures with nano-topographic features are generally well suited to permit miRNA delivery while also being favorable for tissue ingrowth and repair (Stevens and George 2005). The amount of miRNA complexes loaded on the scaffold is also important, so that the addition of miRNA complexes to the scaffold does not have a deleterious effect on mechanical integrity or reduce its cellular attachment/retention properties (Hedberg et al. 2005). Although the low miRNA dose generally applied is unlikely to cause major disruptions, the varied vector types forming the miRNA complexes can significantly impact the viscosity of the starting mixtures for scaffold manufacture. Depending on the target application, the presence of the miRNA complexes may require the corroboration of different structural features. These include mechanical strength of orthopedic graft substitutes (Cui et al. 2014; Zhou et al. 2016; Diao et al. 2015a; Mercado et al. 2016; Wu et al. 2013; Deng et al. 2014b; Sung et al. 2013; Mariner et al. 2012; Diomede et al. 2016; Lolli et al. 2016; Li et al. 2016a) or rheology, viscoelasticity, and physical stimuli responsiveness in the case of hydrogels (Monaghan et al. 2014, 2018; Li et al. 2016b; Cheng et al. 2016; Tan et al. 2017).

Two primary types of scaffold-based miRNA delivery have typically been used: in situ/"cell-free" or ex situ/"cell-mediated" delivery of miRNAs. Based on the nature of their components and their fabrication method, some materials may be better suited for either ex situ or in situ incorporation of miRNA complexes. In the case of *cell-mediated* miRNA delivery, a defined cell population undergoes in vitro miRNA transfection prior to being seeded onto the scaffold of choice. This approach thus necessitates a preliminary in vitro incubation time to obtain the miRNA-modified cells that will become the therapeutic agent delivered by the scaffold. In this way, when the scaffold is implanted in a tissue void, it supports the activity of the miR-modified cells but does not administer more miRNA to any infiltrating cells. In contrast with *cell-mediated* miRNA delivery, the less reported *cell-free* approach does not rely on a preliminary in vitro incubation, denoting an increased "off-the-shelf" potential which might simplify the path to clinical translation (Nguyen et al. 2014; Mariner et al. 2011). In this approach the scaffolds are prepared as depots of miRNA complexes before the cells are seeded or come into contact with the scaffold. These scaffolds can thus be implanted without adding in vitro cultured cells. This way they provide the environment for the infiltrating cells to become transfected with the miRNAs contained in the scaffold and repair the tissue defect (Pelled et al. 2010; Kimelman-Bleich et al. 2011).

A variety of natural or synthetic *hydrogels* have shown ability to deliver miRNA therapeutics using both ex situ and in situ approaches, being the first platforms to

deliver naked miRNA therapeutics (i.e., not complexed with a delivery vector; Monaghan et al. 2014, 2018; Mariner et al. 2011). This simplified concept is likely to provide a quick bolus-like miRNA delivery due to quick diffusion from the biomaterial without further protection from degradation, which would limit treatment efficiency. Natural polymer hydrogels that have proven currently successful for miRNA delivery include the commercial Matrigel™ and formulations of thrombin, silk fibrin, fibrinogen, and alginate (Lolli et al. 2016; Li et al. 2016b; Cheng et al. 2016; Qureshi et al. 2015). Synthetic hydrogels used for miRNA delivery reported to date include an ex situ PEG-norbornene system encapsulating miR-148b mimic + miR-489 inhibitor-modified human MSCs (Mariner et al. 2011), as well as both in situ PEG systems of HyStem-HP™-based delivery of miR-26a mimics (Li et al. 2013) and "on-demand" miR-20a delivery triggered by UV light (Huynh et al. 2016; Nguyen et al. 2014). Another commercial synthetic gel, an amphiphilic triblock copolymer, *Pluronic F127*, was recently employed to mediate the in situ delivery of miR-26a mimic lentiviral complexes (Tan et al. 2017). These are among the biodegradable synthetic materials approved for TERM applications by the US Food and Drug Administration (FDA); however the acidic by-products of their degradation can inhibit tissue repair (Dawes and Rushton 1994). To reduce the detrimental effects of these synthetic polymers, they are often mixed with hyaluronic acid (HyA) among other natural polymers, as found in commercial systems like Glycosan HyStem™.

Interestingly, *electrospun materials* gather most of the miRNA delivery work carried out with non-viral and non-lipid vectors such as the PEI-PEG polyplexes (Zhang et al. 2016a), peptide-chitosan-PEG complexes (Zhou et al. 2016), or silver NPs (Qureshi et al. 2015). Materials like gelatin, or the synthetic PLLA, PCL, and PEG-PCL polymers, are commonly preferred to produce electrospun nanofibrous scaffolds compatible with miRNA delivery (Zhang et al. 2016a; Zhou et al. 2016; Diao et al. 2015a; Qureshi et al. 2015; James et al. 2014). The versatility of the electrospinning methodology explains that these scaffolds have generally been developed as in situ miRNA delivery platforms with great potential as off-the-shelf products. Among the non-electrospun porous spongelike scaffolds, very diverse composition mixtures have been adapted to miRNA delivery, with nearly half of the reports relying on lipid (Eskildsen et al. 2011; Wang et al. 2015; Hoseinzadeh et al. 2016; Vimalraj et al. 2016) and viral delivery (Deng et al. 2013a, b). This collection of work is firmly based on the ex situ methodology of pre-transfecting a target cell population in advance of seeding the scaffolds, thus negating a role for the scaffolds to deliver the miRNA cargo. In clear distinction with this research climate, composites of collagen/nHA developed in the Tissue Engineering Research Group (TERG, Dublin) have been successfully applied to the strategy of cell-free in situ delivery of both miR-mimics and antagomiRs (Mencia Castano et al. 2015, 2016). This work reassures the potential of porous scaffolds to serve as clinically translatable off-the-shelf miRNA delivery platforms. In summary, most existing fabrication processes and biomaterial types appear tunable as vehicles for improved miRNA delivery in combination with either viral or non-viral delivery vectors. Further exploration of the vast biomaterial types available to deliver

miRNAs will provide the next generation of advanced tissue engineering therapeutics; the next section illustrates reported therapeutic potential of some of these strategies.

4 The Role of miRNAs as Modulators of Tissue Repair

miRNAs have a pivotal role in regulating embryonic development (Bartel 2004), and their capacity to induce and modulate the differentiation of pluripotent, multipotent, and progenitor cells, in addition to promoting their accelerated differentiation, holds particular interest for tissue engineering researchers (Levy et al. 2010). Hence, much investigation has concentrated on how specific miRNAs affect different cell types to dictate their function in building and repairing the many tissue types of the body (Weilner et al. 2015, 2016; Feichtinger et al. 2018), in instances with one miRNA influencing several tissue types differently. This knowledge has set the foundation for the later pursuit of miRNA-based tissue engineering approaches (Hackl et al. 2011). This section provides an overview of the known roles of miRNAs by tissue type, while Sect. 5 will discuss how some of these miRNAs have been combined with a wide range of biomaterials, thus beginning to show promise as therapeutics in tissue engineering approaches.

4.1 Bone

The bone is a highly dynamic tissue, with remarkable remodeling and repair capabilities, but unable to heal completely in cases of critical-sized non-union fractures (Martini et al. 2006). The bone repair capability resides in mesenchymal stem cells (MSC), and osteoprogenitors are found in the bone marrow (approx. 1% of its cell population), which replenish the population of osteoblasts and osteocytes via osteogenic differentiation (Aubin 1999). The activation of the osteogenesis process in the bone precursors is thus key to direct bone repair. This can be triggered by many anabolic factors, such as bone morphogenetic proteins (BMPs), which have been pursued as therapeutics with this aim. A selection of over 40 miRNAs have been identified to modulate osteogenesis either positively or negatively (Fig. 7; Hu et al. 2010; Jensen et al. 2010; Li et al. 2008, 2009a, b; Luzi et al. 2008; Mizuno et al. 2008, 2009; Huang et al. 2010; Baglìo et al. 2013; Kim et al. 2009; Kapinas et al. 2009; Schoolmeesters et al. 2009; Clark et al. 2014; Grunhagen and Ott 2013; Eguchi et al. 2013), and miRNA modulation is explored to treat bone defects and diseases such as osteoporosis and osteoarthritis (Hu et al. 2010; Jensen et al. 2010). Many miRNAs of this panel have direct targets that only play a secondary role in the osteogenesis pathway (Kim and Lim 2014), like Crim1/BAMBI, Dickkopft-related proteins (DKK) 1–2, connexin 43, or the focal adhesion kinase (FAK). On the contrary, another set of miRNAs is known to target the main driver of osteogenesis, transcription factor Runx2: miR-133a-3p, miR-204, miR-211, miR-335, and miR-3077-5p (Li et al. 2008; Liao et al.

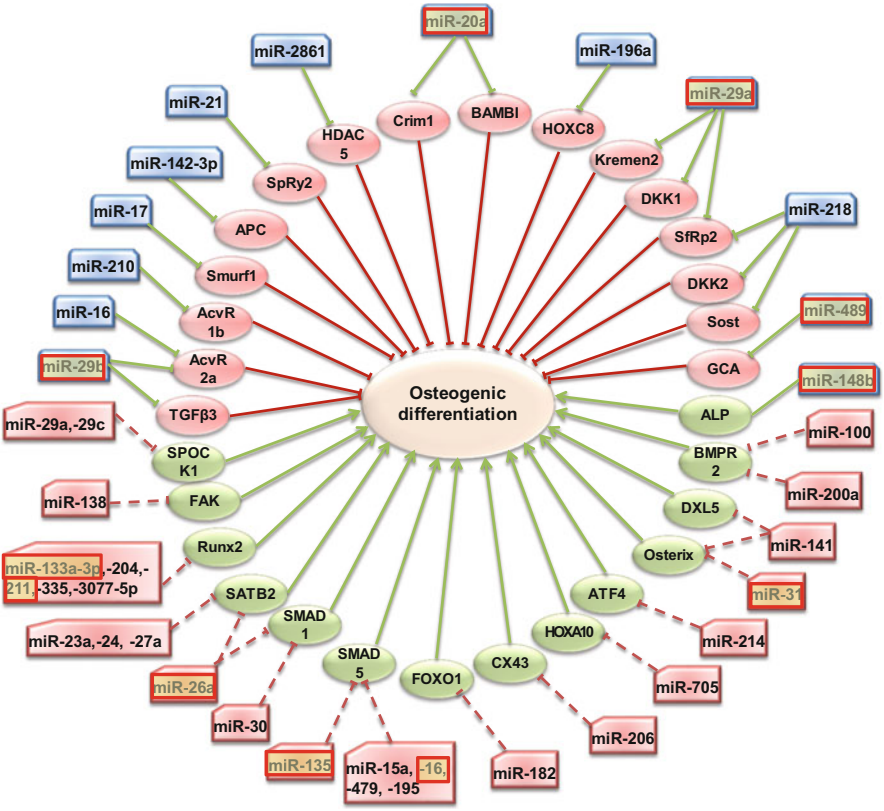


Fig. 7 miRNAs involved in osteogenesis and their validated mRNA targets. Inner circle: red ovals linked to red brake symbols mark proteins that inhibit osteogenesis, green ovals linked to green arrows mark proteins that trigger osteogenesis. Outer circle: miRNAs presented in blue or red are positive or negative regulators of the process, respectively. Highlighting boxes with red border and yellow filling indicate that the miRNA has been assessed as a therapeutic candidate

2013a, b), and another key transcription factor, Osterix, is targeted by miR-31 (Baglìo et al. 2013), miR-141 (Itoh et al. 2009), and miR-214 (Grunhagen and Ott 2013). Currently, about two fifth of the miRNAs identified in this panel have been assessed as therapeutic candidates by the use of miR-mimics for the positive modulators miR-15b (Vimalraj et al. 2016), miR-20a (Huynh et al. 2016), miR-26a (Liao et al. 2014a), miR-29b (Li et al. 2009a), miR-148b (Schoolmeesters et al. 2009), and miR-2861 (Li et al. 2009b) or inhibitors for the negative modulators miR-16 (Mencia Castano et al. 2018), miR-29a (James et al. 2014), miR-31 (Weilner et al. 2016; Deng et al. 2013b), miR-125b (Wang et al. 2017b), miR-133a-3p (Mencia Castano et al. 2016), miR-135 (Li et al. 2008), miR-146a (Xie et al. 2017), miR-221 (Hoseinzadeh et al. 2016), miR-222 (Yoshizuka et al. 2016), miR-214 (Grunhagen and Ott 2013), and miR-489 (Schoolmeesters et al. 2009).

Generally, the therapeutic levels of osteogenesis achieved with these candidates is reported as enhanced mRNA levels of Runx2 and OCN to about threefold the expression of the control group (Li et al. 2009a, b; Kim et al. 2009; Hassan et al. 2012) and modal increases of twofold over untreated cells in alkaline phosphatase (ALP) activity (Schoolmeesters et al. 2009). In terms of calcium deposition, the end-stage marker of functional osteogenesis, non-viral delivery approaches using, for example, miR-148b mimic, incurred ~2-fold increase after 14 days of osteogenic culture (Qureshi et al. 2013), while baculovirus-based delivery has demonstrated 3- to 7-fold increases over the control group (Liao et al. 2014a). Of note, results surpassing all these parameters listed above have recently been reported by the inhibition of both miR-133a-3p and miR-16 using a non-viral delivery system based on nanohydroxyapatite particles (Mencia Castano et al. 2016; Mencia Castano et al. 2018), which denotes exciting possibilities in the realization of miRNA therapeutics for bone repair.

4.2 Cartilage

Cartilage tissue is subclassified histologically into hyaline, elastic, or fibrocartilage. Hyaline cartilage, often referred to as articular cartilage, is found on the articulating surface of joints and is extremely difficult to repair using current tissue engineering methods. This is because articular cartilage is generated and maintained by a population of cells called articular chondrocytes (ACs), which rarely proliferate in adults, functioning primarily to produce extracellular matrix (ECM) components such as glycosaminoglycans (Ulrich-Vinther et al. 2003). Also, the intricate balance of cartilage catabolism and anabolism rates can be disrupted leading to progressive, inflammatory, degenerative lesions of articular cartilage; such is the case of osteoarthritis (OA), a chronic disease ultimately affecting the entire joint. Early treatment of articular cartilage lesions may abrogate disease progression; however suitable treatments with long-term efficacy remain elusive (Huey et al. 2012; Niemeyer et al. 2008; Schnabel et al. 2002; Stenberg et al. 2014). Tissue-engineered alternatives stimulate MSCs to differentiate toward chondrocytes in vitro by modulating several signaling molecules. These include transforming growth factor-β superfamily cytokines (TGFβ, BMPs, GDF5; Johnstone et al. 1998; Legendre et al. 2017; Hatakeyama et al. 2004), the Sox trio – particularly Sox-9 – (Hattori et al. 2010; Lefebvre et al. 2007; Liu and Lefebvre 2015), Wnt/β-catenin signaling (Yuan et al. 2016), as well as histone deacetylase (HDAC) 4, which inhibits chondrocyte hypertrophy, thereby stabilizing the AC population (Huh et al. 2007). Several miRNAs which target the aforementioned factors have also been investigated for their role in promoting chondrogenesis or inhibiting endochondral ossification or inflammation (Fig. 8). In a microarray screening, several miRNAs, including miR-23b, miR-140, and miR-210, were associated with successful chondrogenic differentiation of MSCs, while miR-221, miR-31, and miR-100 were downregulated during chondrogenic differentiation (Gabler et al. 2015). miR-140 is involved in Sox-9-mediated chondrogenesis and is a direct regulator of aggrecanase-2 (Karlsen et al.

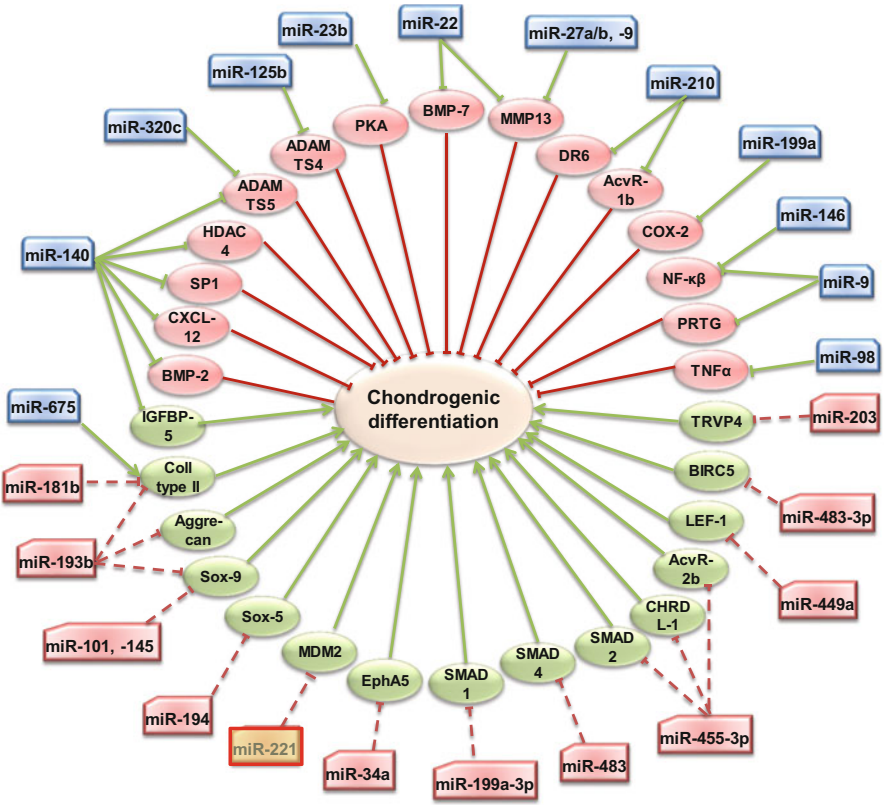


Fig. 8 miRNAs involved in chondrogenesis and their validated mRNA targets. Inner circle: red ovals linked to red brake symbols mark proteins that inhibit chondrogenesis, green ovals linked to green arrows mark proteins that trigger chondrogenesis. Outer circle: miRNAs represented in blue or red are positive or negative regulators of chondrogenesis, respectively. Highlighting boxes with red border and yellow filling indicate that the miRNA has been assessed as a therapeutic candidate

2013, 2016) and thus inhibits excessive degradation of cartilage matrix. miR-455 is also involved in Sox-9-mediated chondrogenesis and has been shown to negatively regulate hypertrophy (Zhang et al. 2015, 2016b), and both miR-140 and miR-455 have been introduced as critical regulators of chondrogenesis (Barter et al. 2015). Gabler et al. demonstrated that miR-181 was associated with MSC chondrogenesis, but the resulting chondrocytes became hypertrophic; no other miRNA candidate could consistently inhibit chondrocyte hypertrophy in this study (Gabler et al. 2015). miR-181 was later confirmed to be non-specific to chondrogenesis (Barter et al. 2015). Other approaches have considered that miRNA modulation of inflammatory genes and proteins may be useful therapeutics for OA, as this is an inflammatory disease. miR-449a expression has been identified in OA chondrocytes which express IL-1β, an inflammatory cytokine. Inhibition of miR-449a showed a protective effect on chondrocytes, inhibiting catabolic gene expression (Park et al. 2016). Other

miRNAs are consistently upregulated in OA patients including miR-27b and miR-22 which inhibit matrix metalloproteinase-13 (MMP-13) production (Akhtar et al. 2010; Iliopoulos et al. 2008) and miR-146a which modulates inflammation by inhibiting NF-κB (Zhao et al. 2011).

4.3 Angiogenesis

Angiogenesis is the process that generates functional vascular networks from already existing vasculature by sprouting or intussusceptive budding, while vasculogenesis generates de novo vasculature within avascular tissues (Balaji et al. 2013). In both processes, endothelial cell differentiation takes place and cells become arranged in tubules with an internal luminal space stabilized by supporting cells like pericytes (Caplan 2008). Both processes are crucial in tissue repair, especially in cases of large or extensive damage, and remain a limiting obstacle for the realization of full-size tissue-engineered grafts of clinical use (Jaklenec et al. 2012). Approaches to develop tissue-engineered vascular networks can focus on either endothelial cell progenitors or supporting cells such as MSCs (Clark et al. 2014; Clarkin and Gerstenfeld 2013). Additionally, natural activators of angiogenesis and vasculogenesis are harnessed as stimuli to further promote the process: low oxygen tissue levels, i.e., hypoxia, which trigger the secretion of vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), angiopoietin (Ang)-1/2, and chemokines like stromal cell-derived factor 1-alpha (SDF1α; Martini et al. 2006; Tayalia and Mooney 2009). All these factors act in an endocrine and paracrine manner to guide the growth, remodeling, and maturation of the newly formed capillary vessels (des Rieux et al. 2011) and can be secreted by both endothelial cells and MSCs. An extended set of miRNAs is currently known to modulate angiogenesis, vasculogenesis, and the pro-angiogenic capacity of MSCs either with a positive or a negative effect (Fig. 9; Li et al. 2013; Suarez and Sessa 2009; Poliseno et al. 2006; Suarez et al. 2007; Fish et al. 2008; Wang et al. 2008; Harris et al. 2008; Fasanaro et al. 2008). Hence, miRNA modulation strategies have arisen to treat angiogenesis-related pathologies such as cardiovascular diseases (van Rooij 2012), diabetes and diabetes-induced retinopathy (Nunes et al. 2015; McArthur et al. 2011; Feng et al. 2011), as well as exacerbated vascularization associated with several cancer types (Ma et al. 2010). Of particular interest for tissue engineering, pro-angiogenic miRNAs that may simultaneously enhance other tissue repair processes, such as promoting the proliferation of progenitor cells, can present additional benefit clinically (Wang and Olson 2009). Some outstanding pro-angiogenic miRNAs considered as tissue engineering therapeutics involve miR-mimics of miR-26a, miR-126, and miR-210 as well as inhibitors of miR-15/16, miR-24, and miR-200b. In particular, a critical role has been shown for miR-126 in developmental studies (Fish et al. 2008) and in coronary ligation infarction models (Wang et al. 2008), while intra-articular injections of atelocollagen containing naked miR-210 mimic accelerated angiogenesis in anterior cruciate ligament (ACL) injuries in vivo (Shoji et al. 2012). Additionally, the systemic delivery of antagomiR-24 and antagomiR-100 improved

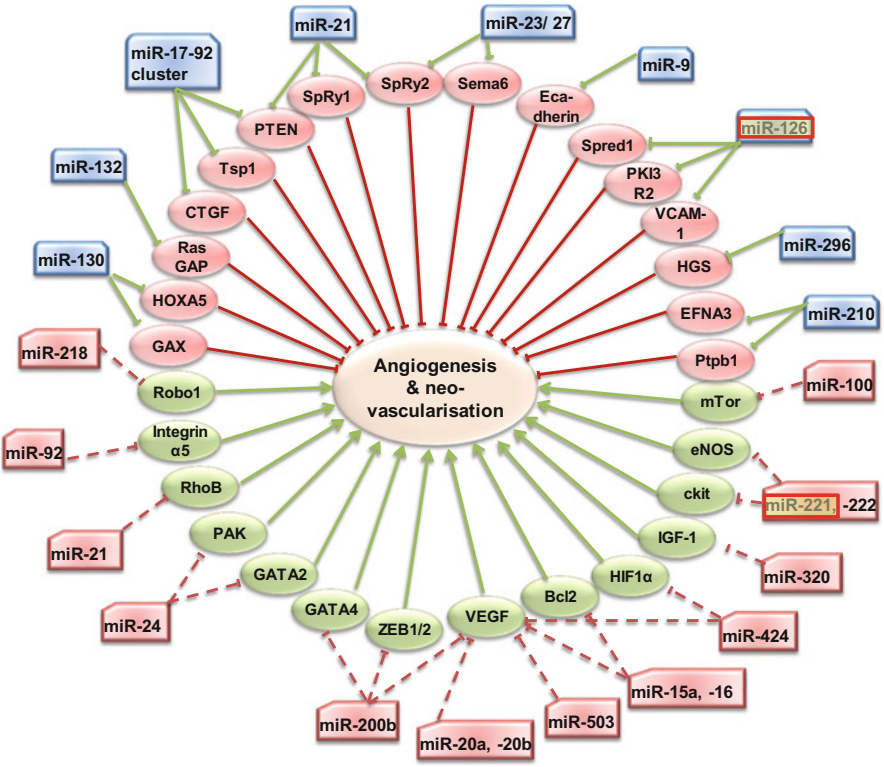


Fig. 9 miRNAs involved in angiogenesis and neovascularization and their validated targets. Inner circle: red ovals linked to red brake symbols mark proteins that inhibit angiogenesis, green ovals linked to green arrows mark proteins that trigger angiogenesis. Outer circle: miRNAs represented in blue or red are positive or negative regulators of the process, respectively (Suarez and Sessa 2009; Poliseno et al. 2006; Suarez et al. 2007; Fish et al. 2008; Wang et al. 2008; Harris et al. 2008; Fasanaro et al. 2008). Highlighting boxes with red border and yellow filling indicate that the miRNA has been assessed as a therapeutic candidate

myocardial angiogenesis in mice myocardial ischemia studies (Grundmann et al. 2011). Contrastingly, therapeutic inhibition of angiogenesis has been shown through the fine-tuning role of miR-200b on multiple growth factors and in epithelial-mesenchymal transition (EMT) in multiple types of cancer, as extensively reviewed by Sinha et al. (Sinha et al. 2015). In a similar line of work, hMSCs were used to deliver miR-16 mimics to prostate cancer xenografts established in mice, and the anti-angiogenic effect of this treatment significantly slowed down tumor growth (Jones et al. 2017). Additionally, subcutaneous implantation – in mice – of micro-vessels engineered by culturing miR-16-transfected endothelial cells into collagen-fibrinogen gels demonstrated effective modulation of angiogenesis in vivo (Chamorro-Jorganes et al. 2011). This heterogeneous body of work highlights the ample possibilities of angiogenesis-modulating miRNAs in tissue engineering applications, including its recently increasing interplay with cancer research, where

anti-angiogenic treatments have begun to show promise in tumor size reduction (Yihai 2003; Fang et al. 2012; Dai and Tan 2015).

4.4 Skin

The skin is a tri-layered tissue forming the largest organ of the body (Tumbar et al. 2004). It has excellent self-repair capacity in injuries limited to the superficial or dermal layer; however deep damage to all three layers, i.e., full-thickness wounds, requires further treatment to heal: excessive scarring must be prevented, and often skin grafts or bioengineered skin equivalents (BSE) are needed (Leblebici et al. 2006). Although currently multiple BSEs are commercially available, the regeneration of fully functional skin is yet to be achieved (Shevchenko et al. 2010). miRNA modulation might offer the necessary control over cell behavior to meet this goal (Miller et al. 2015). A number of cell types are involved in the wound repair process, including epithelial cells or keratinocytes, melanocytes, Langerhans cells, Merkel cells, fibroblasts, basal stem cells, and bulge stem cells – from hair follicles and sebaceous glands (Watt and Jensen 2009). The adequate proliferation of these cells, followed by their deposition of ECM components, must also be accompanied by the re-establishment of a vascular network, mainly within the dermal layer (Blanpain and Fuchs 2009). Following the findings that follicular and epithelial morphogenesis defects occur in mice with globally impaired miRNA biogenesis (i.e., Dicer or Ago KO mice Wang et al. 2012; Andl et al. 2006), many miRNA modulators of skin development and repair have been identified (Fig. 10). These miRNAs can regulate (i) the proliferation and cell cycle of epithelial stem cells and fibroblasts (Yi et al. 2008; Suh et al. 2012), (ii) the production of melanin (Kim et al. 2014), (iii) the follicular development and sebogenesis (Schneider et al. 2013), and very notably (iv) the migration of keratinocytes (Yi et al. 2006; Pastar et al. 2012) and (v) the deposition of ECM by fibroblasts (Madhyastha et al. 2012). In many cases one single miRNA affects different cell types, and can have opposing effects depending on the cell type, as seen with miR-21, miR-24, miR-31, miR-125b, miR-200b, miR-203, or the miR-29 family (Peng et al. 2012). In the case of epithelial stem cells, the relevant miRNAs tend to silence the transcription factor p63 or the fibroblast growth factor receptor 2 (FGFR2) to maintain stemness and proliferation, preventing differentiation (Lena et al. 2008). The control of miRNAs over the function of keratinocytes and fibroblasts relates to fine-tuning of the TGFβ signaling pathway and the control of collagenous ECM deposition (Sundaram et al. 2013; Sun et al. 2008). Despite this extensive knowledge, miRNA therapeutic strategies pursued to date in wound healing have predominantly focused on pro-angiogenic miRNAs, described in the section above. Interesting work focused on miR-7 inhibition to rescue the responsiveness of aging fibroblasts to epidermal growth factor (EGF) and hyaluronan (Midgley et al. 2014); this lack of responsiveness leads to chronic non-healing of wounds among the elderly. The anti-miR-7 treatment demonstrated restored differentiation of fibroblasts to myofibroblasts by enhanced formation of α-SMA fibrils and ~4-fold increased cell mobility in photobleaching studies, overall showing

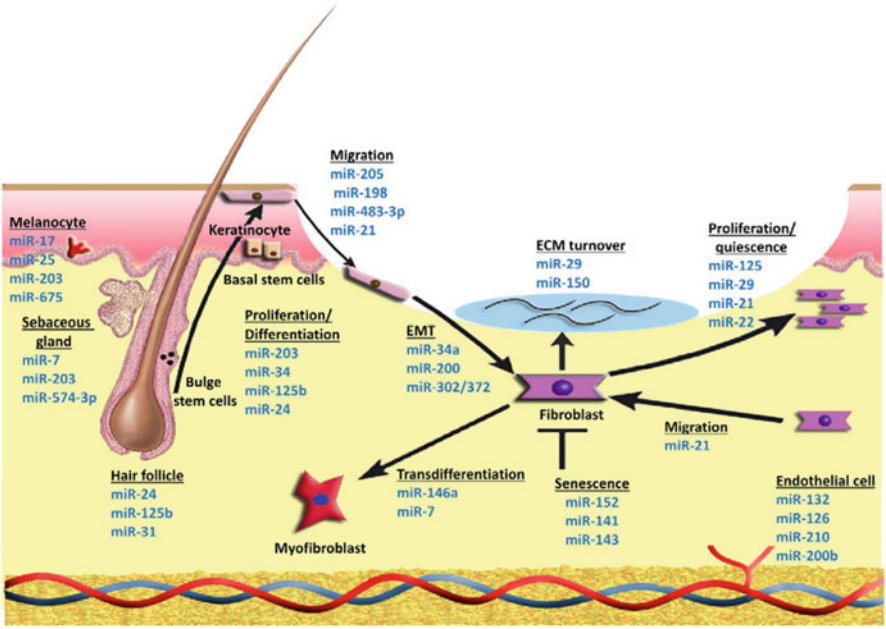


Fig. 10 miRNA modulation of cell behavior in the different skin cell types. (Reproduced with permission Miller et al. (2015); Copyright © 2015 Elsevier B.V. All rights reserved)

promise to prevent chronic wounds. Considering the sound work establishing the role of all these miRNAs in the different cell types found in the skin, we can speculate that the combination of miRNA therapeutics with biomaterials to provide highly functional BSEs is yet to be exploited.

4.5 Muscle

The body is composed of different muscle types, first classified into smooth or striated muscle and then subclassified within striated muscle into the pharyngeal, skeletal, and cardiac muscle types (Cezar and Mooney 2015). Damage or injury to striated muscles includes ischemic heart disease, chronic genetic myopathies, and volumetric muscle loss (VML) injuries, for which muscle-regenerating treatments remain an unmet clinical need (Pascual-Gil et al. 2015). The repair capacity of skeletal muscle is limited by the contribution of resident mononuclear stem cells (or satellite cells) (Lepper et al. 2011), while the limiting factor for cardiac muscle repair is the slow proliferation of adult cardiomyocytes (van Amerongen and Engel 2008). Unsurprisingly, miRNAs have been found to modulate differentiation of satellite cells and myoblasts, with miR-134, miR-296, and miR-470 demonstrating myogenic differentiation of ESCs matching the efficiency previously reported by siRNA silencing of Nanog, Oct4, and Sox-2 (Tay et al. 2008a, b). Hence, the

application of miRNA therapeutics for muscle repair has begun to generate interest, with mimics of miR-26a and miR-206, as well as miR-682 inhibition showing ability to enhance myogenesis after injury (Chen et al. 2011a; McCarthy 2008; Dey et al. 2012). Interestingly, miR-206 can silence multiple myogenesis inhibitors, such as Pax7, Notch3, Igfbp5, connexin 43 (CX43), and histone deacetylase 4 (HDAC4) (McCarthy 2008), denoting a potentially high therapeutic benefit of this miRNA. In line with this, delivery of naked miR-206 mimics along with miR-1 or miR-133 mimics in an atelocollagen mix significantly increased the regeneration of centronucleated myofibers by ~2.4-fold in a rat model of laceration of the tibialis anterior muscle (Nakasa et al. 2010). In addition to myogenesis modulation, miRNA signatures have also been associated with chronic muscle maladies, including Duchenne myodystrophy, sarcopenia, aging-related degeneration, or muscular atrophy (Arashiro et al. 2009; Dimmeler and Nicotera 2013; Wada et al. 2011). For example, aged muscles present decreased levels of miR-181a, while the presence of the miR-15 family members is increased (Mercken et al. 2013). The identification of these expression profiles in patients could serve as a cue to apply personalized miRNA therapeutics to reinstate the balance and normal function of the affected muscles (Feichtinger et al. 2018).

Distinct miRNAs have shown specific potential to improve cardiomyocyte function (van Amerongen and Engel 2008); this includes miR-590 and miR-199a, which halved the infarct size by promoting cardiomyocyte proliferation in a mouse myocardial infarction model (Eulalio et al. 2012). Additionally, miR-302 mimics were able to reduce over 60% cardiac fibrosis size in a period of 50 days (Tian et al. 2015). Meanwhile, inhibiting miR-34a (Boon et al. 2013) or members of the miR-15 family (Porrello et al. 2011), all of which act through arresting the cell cycle of cardiomyocytes, can also result in enhanced cardiomyocyte proliferation and up to 75% less cell death after infarction. Moreover, miRNA modulation can aid cardiac stem cell (CSC) differentiation, as shown by the local injection of miR-499 mimic modified CSCs post-infarction in a mouse model (Wilson et al. 2010; Sluijter et al. 2010). This work demonstrated myocyte mass increase to double that in the control, and improved left ventricular function in the modified CSCs vs. control CSCs, which occurred through targeting Sox-6 and Rod1. In summary, the collective work reported on miRNA-mediated modulation of striated muscle repair suggests an attractive potential to be harnessed in tissue engineering; as seen before, this research area will also rely on the availability of adequately safe and efficient delivery systems to be fully exploited in the near future.

4.6 Neural Tissue

Neural tissue is formed by a number of cell types and has a limited capacity to regenerate after injury, more so in the central nervous system (CNS) than in the peripheral nervous system (PNS) (Schmidt and Leach 2003). Following CNS injury, glial scars are formed which inhibit axonal regeneration and neurogenesis

(GrandPré et al. 2000). Additionally, neurite outgrowth is also inhibited by molecules contained in the CNS myelin (McKerracher et al. 1994). Thus, to recapitulate an environment favorable to neural growth after injury, tissue engineering approaches must promote neurite outgrowth and remyelination while preventing excessive inflammatory reactions and glial scar formation. To achieve successful tissue repair, two divergent avenues are possible: cell replacement therapies and treatments for the local cell population. Because several miRNAs have different effects on various cell types of the neural tissue (Nguyen et al. 2015), treating the local cell population requires neuron or Schwann cell-specific miRNA delivery systems that remain to be developed. Thus, initial efforts have been placed on cell replacement therapies by miRNA-engineering the cells in vitro. A panel of miRNAs modulate dendritogenesis, miR-134 (Christensen et al. 2010); neurogenesis, let 7b/d, miR-34a, miR-125b, miR-137, miR-184, and miR-195 (Nguyen et al. 2015; Stappert et al. 2015; Perruisseau-Carrier et al. 2011); and oligodendrocyte maturation, miR-219 and miR-338 (Svaren 2014). Hence, it is envisaged that miRNA modulation can improve the neuronal cell reprogramming yields that limit this cell replacement strategy. The reprogramming can be targeted to different types of adult stem cells or to trans-differentiate fibroblasts. For example, successful trans-differentiation of mouse embryonic fibroblasts (MEFs) to neuronal-like cells has been shown following lentiviral delivery of miR-9/9* and miR-124 mimics (Yoo et al. 2011; Xue et al. 2013). While both miRNAs can silence the *a* subunit of the BAF chromatin-remodeling complex (BAF53a), they also possess additional targets involved in neurogenesis: miR-9 targets the Hes family members (Bonev et al. 2012), in addition to TLX, a nuclear receptor directing neural stem cell self-renewal (Zhao et al. 2009). Meanwhile, miR-124 is known to also silence a repressor of neuron-specific splicing, that is, polypyrimidine tract binding protein 1 (PTBP1) (Makeyev et al. 2007), and to target multiple elements of Notch signaling (Cheng et al. 2009). This body of work highlights the beneficial effect achieved by the multi-targeting potential of these miRNAs. Other interesting work on miRNAs for neural tissue engineering has shown enhanced neurogenesis with mimics of miR-124a (Hwang et al. 2011), the miR-106b~25 cluster (Brett et al. 2011), or screening of miR-153, miR-181a/a*, miR-324/* (Stappert et al. 2013), and miR-146b, miR-23b, and miR-99a (Stevanato and Sinden 2014). These reports have generally targeted neural stem/progenitor cells (NSPCs) using retrovirus (Brett et al. 2011) or commercial lipid vectors to deliver the miRNAs (Stappert et al. 2013; Stevanato and Sinden 2014), achieving ~2.6-fold increases in the key neuronal marker Tuj1. However, a miR-124 delivery system (Hwang et al. 2011) employed a rabies virus glycoprotein (RVG)-enabled carrier with ability to specifically target nicotinic acetylcholine receptors on neuronal cells, showing significant promise for in vivo miRNA delivery. This delivery system showed 90% reporter silencing efficiency in Neuro2a cells, and combined with mannitol for in vivo administration, it accumulated in the brain after successfully passing the blood-brain barrier. This development thus showed promise as a treatment for the localized cell population and alternative to cell replacement approaches.

5 The Proven Therapeutic Potential of miRNAs to Date

The progress in miRNA therapeutics research for TERM is increasingly proving to have potential for applications in angiogenesis and vasculogenesis, the skin, the cardiovascular system, and orthopedics. From the summary presented in Table 5, it is clear that most work has been done in the area of bone repair; however, positive preclinical results have been reported in viral miRNA delivery for muscle repair (Cheng et al. 2016) and enhanced neurogenesis (Brett et al. 2011). In this highly dynamic field, miRNAs are also being combined with other therapeutics like small chemicals or separate pDNA vectors (Liao et al. 2014a; Shi et al. 2014).

5.1 Bone Healing

The area of bone healing has been the most prolific to date in demonstrating the therapeutic potential of miRNA-based treatments, with a number of reports focusing on the use of miR-26a mimics. These employed varied delivery platforms like Lipofectamine® 2000 on porous HA scaffolds (Wang et al. 2015), PEI polyplexes on nanofibrous PLLA scaffolds (Zhang et al. 2016a), or the siPORT NeoFx™ on HyStem-HP hydrogels (Li et al. 2013). The interest on miR-26a began by investigating a pro-angiogenic effect seen through BMP2/Drosophila mothers against decapentaplegic (SMAD) signaling (Icli et al. 2013) and the p38-MAPK/VEGF pathway (Zuo et al. 2015). However, a direct effect of miR-26a on modulating osteoblast activity has more recently been proven through targeting glycogen synthase kinase-3beta (GSK-3beta; Zhang et al. 2016a). The current therapeutic effects derived from the miR-26a mimic treatments include the repair of bone defects 12 weeks after implantation (Li et al. 2013; Wang et al. 2015). Additionally the cell-free system by Zhang et al., encompassing the intermediate polyplex encapsulation in scaffold-immobilized PLGA microspheres, increased bone healing in both healthy and osteoporotic mice just 8 weeks after implantation (Zhang et al. 2016a).

miR-148b mimics consistently demonstrated increased ALP activity and were used on PEG-norbornene scaffolds (Mariner et al. 2012) and cell-seeded baculovirus-/gelatin-coated PLGA (Liao et al. 2014b) and Ag NP/PCL (Qureshi et al. 2015) scaffolds, before the BMP2 inhibitor *NOG* was identified as its direct target (Li et al. 2016). In the first two of these studies, miR-148b was co-delivered with miR-489 inhibitors or BMP2 pDNA, respectively. The baculovirus systems enhanced healing by ~80% 12 weeks post-implantation (Liao et al. 2014b; Li et al. 2016). Comparatively, the non-viral Ag NP/PCL system reported ~32% increased healing, although this platform was successfully controlled by photoactivation, an elegant development to confer temporal control and improved safety to this proposed bone graft substitute (Qureshi et al. 2015). Non-viral delivery of miR-15b or miR-20a mimics to MSCs has also shown significant potential to enhance therapeutic osteogenesis in vitro (Huynh et al. 2016; Nguyen et al. 2014; Vimalraj et al. 2016). Specifically, miR-15b modulation induced paracrine osteogenesis on osteoblasts using conditioned medium from mMSCs that were transfected using the

X-tremeGENE agent, with a greater effect when MSCs were cultured on the carboxymethyl cellulose-nHA scaffolds (Vimalraj et al. 2016). The work performed with miR-20a mimics first used PEI polyplexes and in situ gelling PEG hydrogels co-delivering Noggin siRNA (Nguyen et al. 2014), to later incorporate on-demand release using photodegradation triggered by UV light (Huynh et al. 2016).

Lentivirus systems pre-modifying ASCs with miR-135 mimics (Xie et al. 2016) or inhibitors of miR-146a (Xie et al. 2017), and miR-31 (Deng et al. 2013b, 2014a, b) have demonstrated relevant potential to modulate osteogenesis and bone healing as cell-based approaches. In the lentivirus systems applied to poly(sebacoyl diglyceride) (PSeD) scaffolds, the treatments significantly enhanced ASC-directed bone regeneration, while counter-treatments with miR-135 inhibitors or miR-146a mimics reverted the therapeutic effect (Xie et al. 2016, 2017). Works on the lentiviral miR-31 inhibitory strategy have shown significant bone repair in both rat calvarial defects (Deng et al. 2013b, 2014a) and canine orbital defects (Deng et al. 2014b). This work underlined a feedback mechanism activated by BMPs and leading to miR-31 suppression. Finally, demineralized bone matrices (DBMs) were combined with MSCs pre-modified by lentiviral miR-125b inhibition targeting the BMP type-1b receptor (BMPR1b), leading to ~50% higher bone mineral density and maturity (Wang et al. 2017b). Multiple non-viral approaches to miRNA inhibitor delivery have also shown bone repair to date. The ADSC-based application of antagomiR-221 complexes with the commercial agent Lipofectamine® 2000 on nHA/PCL scaffolds proved successfully increased osteogenesis while highlighting differences between the monolayer and scaffold settings (Hoseinzadeh et al. 2016). Meanwhile, the commercial TKO transfection agent was applied to cell-free antagomiR-29a delivery within gelatin nanofibers to target several collagens as well as osteonectin, significantly increasing the synthesis of ECM proteins, although mineralization data were not provided (James et al. 2014).

A growing body of work using cell-free non-viral miRNA delivery platforms has focused on nHA particles as delivery vectors added to porous collagen-nHA scaffolds first designed for bone repair applications (Mencia Castano et al. 2015, 2016). The scaffold system has shown promising functionality at ~20% silencing with miR-mimic reporters and 88.4% inhibition with antagomiR reporters (Mencia Castano et al. 2015); these scaffolds delivering antagomiR-16 and antagomiR-133a demonstrated increased osteogenesis by hMSCs to levels seen only with viral systems (Mencia Castano et al. 2018; Li et al. 2008) and are currently being pursued in preclinical studies. Among preclinical reports, gelatin/spongostan scaffolds carrying naked miR-214 inhibiting sponges pre-transfected in ovariectomized (OVX) MSCs increased osteoporotic bone healing, especially when co-delivered with plasmid BMP2 (Li et al. 2016a), while a naked antagomiR-222 in an atelocollagen mix lead to successful refractory fracture healing after 8 weeks (Yoshizuka et al. 2016).

5.2 Cartilage Healing

In comparison with the increasing understanding of how miRNA modulation can enhance in vitro chondrogenesis, realization of miRNA therapeutics for enhanced

cartilage healing is noticeably delayed. This is partly because single or focal defects present difficulties of low cell density and avascularity and partly because degenerative cartilage damage resulting from osteoarthritis (OA) or advanced tear of the anterior cruciate ligament (ACL) is greatly affected by impaired functions of the immune system. Recently reported in vitro studies of 3D pellet cultures without the support of a biomaterial scaffold have demonstrated the fine-tuning and space-dependent effect of different miRNA therapeutic candidates. This work entails the inhibition of miR-374, miR-379, or miR-503 with Lipofectamine® 2000 complexes in rat primary chondrocytes (Jee et al. 2018), as well as the lentiviral delivery of miR-140 mimics administered in combination with TGFβ3 supplements to human-induced pluripotent stem cells (Mahboudi et al. 2018). The thorough zonal study of growth plate chondrocytes presented by Jee and colleagues (2018) underscored a parallel between the concentration of parathyroid hormone-related protein (PTHrP) and the three miRNAS: miR-374, miR-379, and miR-503. High levels of all PTHrP and three miRNAs enhanced chondrocyte proliferation while inhibiting hypertrophy, but low levels of these directed hypertrophic differentiation. Mahboudi et al. showed enhanced aggrecan and collagen type II expression in the pellets after 21 days, validating the pro-chondrogenic role of miR-140 (Mahboudi et al. 2018); however, collagen type X expression was also enhanced with the miRNA treatment, indicative of the undesired hypertrophy that leads to dysfunctional calcification of cartilage.

Based on these reports, miRNA-based approaches for cartilage tissue engineering are still envisaged to involve the delivery of miRNA-modified cells within biomaterials. There has only been one successful in vivo report on cartilage regeneration, which demonstrated >95% successful inhibition of miR-221 in pellet culture and later incorporated the miR-221 inhibited cells in a hydrogel suspension of alginate injected within osteochondral biopsies (Lolli et al. 2016). This miR-221 silencing system used Lipofectamine RNAiMAX to transfect hMSCs and was introduced by Lolli et al. in their previous in vitro work showing the increase of chondrogenic markers with this therapeutic in the absence of TGFβ (Lolli et al. 2014). These complex constructs were then inserted into subcutaneous pockets in the back of immunosuppressed mice and lead to enhanced deposition of cartilaginous matrix within the constructs at a 12-week time-point. Moreover, the study assessed the hallmark indicator of hypertrophy, collagen type X, and did not find evidence of expression in the miR-221 silenced group. These results point to an exciting direction to provide alternatives to the limitedly successful options of chondroplasty/mosaicplasty, microfracture, or articular replacement.

5.3 Myogenesis and Myocardium Repair

Similarly to the landscape of cartilage repair, the pursuit of miRNA therapeutics in the area of myocyte functional regeneration remains in its infancy. However, progress in this arena appears valuable not only to address diseased or injured muscles but also as in vitro models for drug testing in the effort to reduce animal testing. To date, miRNA modulation has successfully augmented contractile force of human

skeletal myoblasts grown on fibrin-based hydrogels via delivery of miR-133a and miR-696 inhibitors with the commercial agent siPORT NeoFx™ (Cheng et al. 2016). This recent work by Cheng et al. demonstrated that inhibition of both miRs could enhance the peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) among other markers, indicating increased myogenesis of the myobundles after 2 weeks in static 3D culture. Cardiomyocyte function modulation has also been reported with miRNA treatments attempting to restore cardiovascular function after myocardium damage. With the premise of recovering cardiomyocyte function and enhancing their proliferation rate, the complications generated by insufficient angiogenesis could be somewhat prevented (van Amerongen and Engel 2008). This was shown using a mouse ischemia/reperfusion model where the animals received myocardial injections of a Glycosan HyStem/hyaluronan hydrogel carrying naked miR-29b mimics (Monaghan et al. 2018). The treatment enhanced the de novo deposition of collagen fibers bordering the infarct zone and improved scar remodeling and vascularity, ultimately preserving the myocardial function and increasing the cardiac ejection fraction by ~10%. In another elegant approach to therapeutically enhance the functional population of cardiomyocytes, neonatal cardiac fibroblasts were successfully reprogrammed with a multi-miRNA mimic treatment of miR-1, miR-133, miR-208, and miR-499, delivered using the commercial agent DharmaFECT and fibrinogen-based hydrogels as the support biomaterial (Li et al. 2016b). The combination of the treatment with the 3D hydrogel culture improved – over the 2D culture results – the yield of successfully reprogrammed cardiomyocytes through a MMP-dependent mechanism. The realization of injectable biomaterials adapted for successful delivery of miRNA therapeutics, such as these hydrogels, can provide an attractive route for noninvasive regenerative treatment of myocardial dysfunctions and ischemic diseases.

5.4 Angiogenesis, Vasculogenesis, and Wound Healing

Angiogenesis is a crucial process not only for the homeostasis and repair of the circulatory system but also for the successful repair of most tissue types, with the exception of cartilage healing. A recent application of miRNA therapeutics for vascular tissue engineering was introduced by delivering miR-126 mimics to vascular endothelial cells using multi-modified peptide/chitosan/PEG NPs (Zhou et al. 2016). The miR-chitosan complexes were embedded in the innermost layer of a fibrous electrospun composite based on PEG, PLL, PCL, and gelatin (PELCL) and, through silencing SPRED-1, effectively enhanced cell proliferation and in vivo endothelialization. Importantly, exacerbated angiogenesis is a leading cause of tumor growth, and numerous anti-angiogenic treatments have been proposed to inhibit tumor growth. With the use of 3D biomaterials emerging in cancer research, a number of reports have already explored miRNAs in tissue-engineered approaches to control vascular hyperplasia. To this end, fragments of jugular vein enhanced with a lentiviral miR-26a mimic and Pluronic F127 hydrogel mix reduced venous neointimal hyperplasia of vascular smooth muscle cells in a rat model (Tan et al. 2017).

This treatment worked by inhibiting cell proliferation through silencing the target MAPK6, and also reducing the intima-to-media ratio, reducing the neointimal hyperplasia by ~30%. Similarly, the Pluronic F127 hydrogel carrying adenoviral miR-221 inhibiting sponge mixtures also hindered cell proliferation and neointimal formation in the rat vein grafting model, with reduced neointima/media ratio, neointimal area, and thickness (Wang et al. 2016). Additionally, this study also determined improved hemodynamics and related these effects with the targeting of Kip1. Taking this body of work together, miRNA modulation appears as a significant promise to bypass failure in vein grafting procedures.

Delivery of naked miR-29b mimics from a collagen-based scaffold has been used in the area of wound healing, with the aim of directing beneficial remodeling of the ECM while preventing the formation of scar tissue (Monaghan et al. 2014). The scaffolds were first tested in vitro culturing primary fibroblasts, and the miR-29b mimics released from this platform showed beneficial ~40% and ~15% reductions of collagen type I and type III levels at the 2-week time-point. Following these observations, the scaffolds were implanted in full-thickness skin excisional wounds in rats and achieved dose-dependent improvements in the ratios of collagen type III/I and matrix metalloproteinase (MMP)-8/tissue inhibitor of metalloproteinase (TIMP)-1, as well as reducing the contraction of the wound.

5.5 Neurogenesis and Neural Tissue Healing

With the limited clinical options currently available to treat neurological pathologies and improve nerve healing, miRNA therapeutics might have significant potential for neural tissue engineering. Of note, various 3D scaffold systems have been used to better elucidate the miRNA mechanisms regulating the self-renewal vs. differentiation fate of neural stem cells, in comparison with the traditional monolayer culture. To do so, Cui et al. employed spongy collagen-based scaffolds together with PA-1 cells and neural stem cells (NSCs), reporting differential miRNA profiles in 3D culture and decreased NSC differentiation (Cui et al. 2014). This effect was associated with the reduced expression of a panel of miRNAs with validated targets involved in NSC fate decision, including miR-20. Additionally, NSCs differentiated on nanofibrous scaffolds of aminopropyl-silica have been reported to enhance the levels of let-7 miRNAs (Mercado et al. 2016), whose decisive control of NSC differentiation had previously been reported (Brett et al. 2011). Building on the physiologically relevant data obtained from these studies of 3D-cultured neural progenitors, three different miRNAs have been assessed as therapeutic candidates in this nascent field: miR-222 mimics and miR-219 and miR-338 inhibitors. The therapeutic potential of miR-219 and miR-338 inhibitors, complexed with TransIT-TKO, to enhance the development of oligodendroglial precursor cells (OPC) was assessed in vitro in topographically controlled electrospun PCL scaffolds (Diao et al. 2015a, b). Both antagomiR treatments significantly enhanced OPC maturation and remyelination, and this was favored by the topography (Diao et al. 2015b). More recently, micellar NPs delivering miR-222 mimics within an aligned nanofibrous

hydrogel based on collagen, PCL, and *co*-ethylethylene phosphate have shown therapeutic promise in a rat spinal cord injury model (Nguyen et al. 2017). This work demonstrated axonal regeneration with adequate alignment just 1 week after injury induction while finding minimal scar tissue formation and inflammation and represents an attractive development for advanced neural tissue repair.

6 Future Outlook

The discovery of miRNAs and the recent explorations of their therapeutic potential have widely impacted the landscape of gene therapy. This impact has been more prominent in the areas of genetic disorders (i.e., single gene mutations) and oncology, with research only recently beginning to focus on the field of TERM and concentrating on establishing the role of miRNAs in tissue development and repair. The fruition of this knowledge now opens up vast and novel therapeutic opportunities, propelling a research area that has grown exponentially in the last 5–8 years. Perhaps the most immediate opportunity exists on the application of miRNAs in the areas of biomarkers and personalized medicine (Hackl et al. 2016); miRNA profiles of individual patients may serve both to prevent and diagnose diseases and to monitor the patient response to the assigned treatments. The use of miRNAs as biomarkers accounts for the highest number of clinical trials currently ongoing (Ginn 2018). Nonetheless, the application of miRNAs as therapeutics remains very appealing, as their chemical manipulation and incorporation into efficient delivery systems can be facilitated by their low molecular weight and small size, in comparison with plasmid DNA or proteins. More importantly, miRNAs have the potential to interact with multiple targets and thus regulate biological processes at several points. Despite limitations with clinical translation related to issues such as pharmacokinetic and intracellular delivery obstacles, commercialization of miRNA products is increasing all the time, and many companies have taken stakes in the clinical application of miRNAs. For example, Santaris Pharma, Regulus, miRagen, InteRNA Tech., Mirna Therapeutics, and EnGeneIC are currently competing with the giant of RNA therapeutics, Moderna, and have led a number of miRNA therapeutics to enter clinical trials, progressing up to Phase II. This progress strengthens the idea that miRNA treatments bear a bright future and the capacity to make an impact in the generation of the next-generation TERM therapeutics.

7 Conclusions

This chapter has reviewed the wide range of techniques and research tools currently available for miRNA exploration. While these currently allow the investigation of miRNAs from the discovery of novel units to the understanding of their physiopathological roles, research efforts need to make more accessible the use of high-throughput RNAseq. Specific focus must be placed in developing new benchtop equipment, as well as better computational and systems biology tools, to simplify the storage of the

complex and large datasets that this technique generates, permit their quick visualization, and facilitate the identification of novel interactions. In addition, genetically modified animal models that suppress the expression of a particular miRNA are currently underdeveloped in comparison with the tools to generate models overexpressing the miRNA of interest. The application of CRISPR technology to generate miR-specific and tissue- and cell-specific KO animal models will allow an improved understanding of the importance of anomaly low levels of a miRNA of interest for the normal function of a given tissue type or organ. This enhanced knowledge will serve to test better and more accurately the novel miRNA modulation strategies and TE systems surfacing in the field. Ultimately the biggest research need for the use of miRNA therapeutics in TERM is invariably the development, availability, and standardization of safe, noninvasive, and marketable biomaterial-based delivery systems of high quality. These systems must serve the main goal of enhancing the miRNA bioavailability in a spatiotemporally controlled manner and reducing the undesired uptake by nontarget tissues, which would occur when employing systemic routes of administration. Some of the key factors which might support the potential of miRNA applications in tissue engineering and regenerative medicine include accumulating translatable knowledge gained from siRNA and anti-tumor miRNA-based therapies, where translation toward the clinic is at a more advanced stage of development, as well as exploiting the highly innovative systems for biomaterial-based miRNA delivery. If these developments are accompanied by extensive advances at the regulatory level, the field of biomaterial-based miRNA delivery will likely begin the transition from bench to bedside within the next decade.

Acknowledgments Work supported by the European Research Council (ERC) Grant Agreement n° 239685 and 665777 and the Science Foundation Ireland (SFI) funded AMBER Centre.

References

(Agency), E.E.M (2016a) Strimvelis autologous CD34+ enriched cell fraction that contains CD34+ cells transduced with retroviral vector that encodes for the human ADA cDNA sequence. EMA/CHMP/249031/2016: European Medicines Agency

(Agency), E.E.M (2016b) Zalmoxis allogeneic T cells genetically modified with a retroviral vector encoding for a truncated form of the human low affinity nerve growth factor receptor (ΔLNGFR) and the herpes simplex I virus thymidine kinase (HSV-TK Mut2). EMA/454627/2016: European Medicines Agency

Akhtar N, Rasheed Z, Ramamurthy S, Anbazhagan AN, Voss FR, Haqqi TM (2010) MicroRNA-27b regulates the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes. Arthritis Rheum 62:1361

Akhtar MM, Micolucci L, Islam MS, Olivieri F, Procopio AD (2016) Bioinformatic tools for microRNA dissection. Nucleic Acids Res 44:24

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell, 4th edn. Garland Science, New York

Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, andTuschl T (2003) A uniform system for microRNA annotation. RNA 9:277

Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, Andl CD, Seykora JT, Hannon GJ, Millar SE (2006) The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. Curr Biol 16:1041

Arashiro P, Eisenberg I, Kho AT, Cerqueira AM, Canovas M, Silva HC, Pavanello RC, Verjovski-Almeida S, Kunkel LM, Zatz M (2009) Transcriptional regulation differs in affected facioscapulohumeral muscular dystrophy patients compared to asymptomatic related carriers. Proc Natl Acad Sci U S A 106:6220

Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T (2003) The small RNA profile during *Drosophila melanogaster* development. Dev Cell 5:337

Aubin JE (1999) Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem 72:396

Bader AG, Brown D, Winkler M (2010) The promise of microRNA replacement therapy. Cancer Res 70:7027

Baglìo SR, Devescovi V, Granchi D, Baldini N (2013) MicroRNA expression profiling of human bone marrow mesenchymal stem cells during osteogenic differentiation reveals Osterix regulation by miR-31. Gene 527:321

Balaji S, King A, Crombleholme TM, Keswani SG (2013) The role of endothelial progenitor cells in postnatal vasculogenesis: implications for therapeutic neovascularization and wound healing. Adv Wound Care (New Rochelle) 2:283

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281

Barter MJ, Tselepi M, Gómez R, Woods S, Hui W, Smith GR, Shanley DP, Clark IM, Young DA (2015) Genome-wide MicroRNA and gene analysis of mesenchymal stem cell chondrogenesis identifies an essential role and multiple targets for miR-140-5p. Stem Cells (Dayton, Ohio) 33:3266

Basyuk E, Suavet F, Doglio A, Bordonne R, Bertrand E (2003) Human let-7 stem-loop precursors harbor features of RNase III cleavage products. Nucleic Acids Res 31:6593

Beavers KR, Nelson CE, Duvall CL (2014) MiRNA inhibition in tissue engineering and regenerative medicine. Adv Drug Deliv Rev

Behr J-P (1997) The proton sponge: a trick to enter cells the viruses did not exploit. CHIMIA Int J Chem 51:34

Benes V, Castoldi M (2010) Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods 50:244

Bernardo BC, Charchar FJ, Lin RCY, McMullen JR (2012) A MicroRNA guide for clinicians and basic scientists: background and experimental techniques. Heart, Lung Circ 21:131

Bhattacharya A, Ziebarth JD, Cui Y (2014) PolymiRTS database 3.0: linking polymorphisms in microRNAs and their target sites with human diseases and biological pathways. Nucleic Acids Res 42:D86

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

Bonadio J, Smiley E, Patil P, Goldstein S (1999) Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med 5:753

Bonev B, Stanley P, Papalopulu N (2012) MicroRNA-9 modulates Hes1 ultradian oscillations by forming a double-negative feedback loop. Cell Rep 2:10

Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, Kaluza D, Tréguer K, Carmona G, Bonauer A, Horrevoets AJ, Didier N, Girmatsion Z, Biliczki P, Ehrlich JR, Katus HA, Müller OJ, Potente M, Zeiher AM, Hermeking H, Dimmeler S (2013) MicroRNA-34a regulates cardiac ageing and function. Nature 495:107

Bose S, Tarafder S (2012) Calcium phosphate ceramic systems in growth factor and drug delivery for bone tissue engineering: a review. Acta Biomater 8:1401

Boudreau RL, Martins I, Fau-Davidson BL, Davidson BL (2009) Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. Mol Ther 17:169

Braasch DA, Corey DR (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol 8:1

Brett JO, Renault VM, Rafalski VA, Webb AE, Brunet A (2011) The microRNA cluster miR-106b~25 regulates adult neural stem/progenitor cell proliferation and neuronal differentiation. Aging (Albany NY) 3:108

Bucholz RW (2002) Nonallograft osteoconductive bone graft substitutes. Clin Orthop Relat Res 395:44

Budak H, Bulut R, Kantar M, Alptekin B (2016) MicroRNA nomenclature and the need for a revised naming prescription. Brief funct genomics. The Author 2015. Published by Oxford University Press For permissions, please email: journals.permissions@oup.com, England, p 65

Caplan AI (2008) All MSCs are pericytes? Cell Stem Cell 3:229

Caporali A, Emanueli C (2011) MicroRNA-503 and the extended microRNA-16 family in angiogenesis. Trends Cardiovasc Med 21:162

Caudy AA, Myers M, Hannon GJ, Hammond SM (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev 16:2491

Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, Tops BB, Silva JM, Myers MM, Hannon GJ, Plasterk RH (2003) A micrococcal nuclease homologue in RNAi effector complexes. Nature 425:411

cBio (2010) MicroRNA.org. In: M.S.-K.C.C.M (ed) Computational biology center. Memorial Sloan-Kettering Cancer Center ™, New York

Cezar CA, Mooney DJ (2015) Biomaterial-based delivery for skeletal muscle repair. Adv Drug Deliv Rev 84:188

Chamorro-Jorganes A, Araldi E, Penalva LOF, Sandhu D, Fernandez-Hernando C, Suarez Y (2011) MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. Arterioscler Thromb Vasc Biol 31:2595

Chen CZ, Li L, Lodish HF, Bartel DP (2004) MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83

Chen Y, Gelfond J, McManus LM, Shireman PK (2011a) Temporal microRNA expression during in vitro myogenic progenitor cell proliferation and differentiation: regulation of proliferation by miR-682. Physiol Genomics 43:621

Chen CL, Luo WY, Lo WH, Lin KJ, Sung LY, Shih YS, Chang YH, Hu YC (2011b) Development of hybrid baculovirus vectors for artificial MicroRNA delivery and prolonged gene suppression. Biotechnol Bioeng 108:2958

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

Cheng CS, Ran L, Bursac N, Kraus WE, Truskey GA (2016) Cell density and joint microRNA-133a and microRNA-696 inhibition enhance differentiation and contractile function of engineered human skeletal muscle tissues. Tissue Eng Part A 22:573

Chew SY (2015) MicroRNAs in tissue engineering & regenerative medicine. Adv Drug Deliv Rev 88:1

Christensen M, Larsen LA, Kauppinen S, Schratt G (2010) Recombinant adeno-associated virus-mediated microRNA delivery into the postnatal mouse brain reveals a role for miR-134 in dendritogenesis in vivo. Front Neural Circ 3:16

Christopher AF, Kaur RP, Kaur G, Kaur A, Gupta V, Bansal P (2016) MicroRNA therapeutics: Discovering novel targets and developing specific therapy. Perspect Clin Res 7:68

Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102:13944

Clark EA, Kalomoiris S, Nolta JA, Fierro FA (2014) Concise review: microRNA function in multipotent mesenchymal stromal cells. Stem Cells 32:1074

Clarkin CE, Gerstenfeld LC (2013) VEGF and bone cell signalling: an essential vessel for communication? Cell Biochem Funct 31:1

ClinicalTrials.gov (2013) Multicenter phase I study of MRX34, MicroRNA miR-RX34 Liposome Injectable Suspension. October 28, 2013 ed: U.S. National Institutes of Health
Conde J, Oliva N, Atilano M, Song HS, Artzi N (2016) Self-assembled RNA-triple-helix hydrogel scaffold for microRNA modulation in the tumour microenvironment. Nat Mater 15:353
Cui Y, Xiao Z, Chen T, Wei J, Chen L, Liu L, Chen B, Wang X, Li X, Dai J (2014) The miR-7 identified from collagen biomaterial-based three-dimensional cultured cells regulates neural stem cell differentiation. Stem Cells Dev 23:393
Cunniffe GM, O'Brien FJ, Partap S, Levingstone TJ, Stanton KT, Dickson GR (2010) The synthesis and characterization of nanophase hydroxyapatite using a novel dispersant-aided precipitation method. J Biomed Mater Res A 95:1142
Cunniffe GM, Curtin CM, Thompson EM, Dickson GR, O'Brien FJ (2016) Content-dependent osteogenic response of nanohydroxyapatite: an in vitro and in vivo assessment within collagen-based scaffolds. ACS Appl Mater Interfaces 8:23477
Curtin CM, Cunniffe GM, Lyons FG, Bessho K, Dickson GR, Duffy GP, O'Brien FJ (2012) Innovative collagen nano-hydroxyapatite scaffolds offer a highly efficient non-viral gene delivery platform for stem cell-mediated bone formation. Adv Mater 24:749
Curtin CM, Mencía Castaño I, O'Brien FJ (2017) Scaffold-based microRNA therapies in regenerative medicine and cancer. Adv Healthc Mater 7:1700695
Dai X, Tan C (2015) Combination of microRNA therapeutics with small-molecule anticancer drugs: mechanism of action and co-delivery nanocarriers. Adv Drug Deliv Rev 81:184
Dawes E, Rushton N (1994) The effects of lactic acid on PGE2 production by macrophages and human synovial fibroblasts: a possible explanation for problems associated with the degradation of poly(lactide) implants? Clin Mater 17:157
Deiters A (2010) Small molecule modifiers of the microRNA and RNA interference pathway. AAPS J 12:51
Deng Y, Zhou H, Zou D, Xie Q, Bi X, Gu P, Fan X (2013a) The role of miR-31-modified adipose tissue-derived stem cells in repairing rat critical-sized calvarial defects. Biomaterials 34:6717
Deng Y, Wu S, Zhou H, Bi X, Wang Y, Hu Y, Gu P, Fan X (2013b) Effects of a miR-31, Runx2, and Satb2 regulatory loop on the osteogenic differentiation of bone mesenchymal stem cells. Stem Cells Dev 22:2278
Deng Y, Bi X, Zhou H, You Z, Wang Y, Gu P, Fan X (2014a) Repair of critical-sized bone defects with anti-miR-31-expressing bone marrow stromal stem cells and poly(glycerol sebacate) scaffolds. Eur Cell Mater 27:13
Deng Y, Zhou H, Gu P, Fan X (2014b) Repair of canine medial orbital bone defects with miR-31-modified bone marrow mesenchymal stem cells. Invest Ophthalmol Vis Sci 55:6016
des Rieux A, Ucakar B, Mupendwa BP, Colau D, Feron O, Carmeliet P, Preat V (2011) 3D systems delivering VEGF to promote angiogenesis for tissue engineering. J Control Release 150:272
Desvignes T, Batzel P, Berezikov E, Eilbeck K, Eppig JT, McAndrews MS, Singer A, Postlethwait JH (2015) microRNA nomenclature: a view incorporating genetic origins, biosynthetic pathways, and sequence variants. Trends Genet: TIG 31:613
Dey BK, Gagan J, Yan Z, Dutta A (2012) miR-26a is required for skeletal muscle differentiation and regeneration in mice. Genes Dev 26:2180
Diao HJ, Low WC, Lu QR, Chew SY (2015a) Topographical effects on fiber-mediated microRNA delivery to control oligodendroglial precursor cells development. Biomaterials 70:105
Diao HJ, Low WC, Milbreta U, Lu QR, Chew SY (2015b) Nanofiber-mediated microRNA delivery to enhance differentiation and maturation of oligodendroglial precursor cells. J Control Release 208:85
Dimmeler S, Nicotera P (2013) MicroRNAs in age-related diseases. EMBO Mol Med 5:180
Diomede F, Merciaro I, Martinotti S, Cavalcanti MF, Caputi S, Mazzon E, Trubiani O (2016) miR-2861 is involved in osteogenic commitment of human periodontal ligament stem cells grown onto 3D scaffold. J Biol Regul Homeost Agents 30:1009
Ebert MS, Sharp PA (2010) MicroRNA sponges: progress and possibilities. RNA 16:2043

Ebert MS, Neilson JR, Sharp PA (2007) MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4:721

Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR (2018) Gene therapy clinical trials worldwide to 2017: An update. J Gene Med 20:e3015

Eguchi T, Watanabe K, Hara ES, Ono M, Kuboki T, Calderwood SK (2013) OstemiR: a novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesencymal stem cells. PLoS One 8:e58796

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, andTuschl T (2001a) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J 20:6877

Elbashir SM, Lendeckel W, Tuschl T (2001b) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188

Eskildsen T, Taipaleenmaki H, Stenvang J, Abdallah BM, Ditzel N, Nossent AY, Bak M, Kauppinen S, Kassem M (2011) MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. Proc Natl Acad Sci U S A 108:6139

Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M (2012) Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492:376

Evans CH (2013) Advances in regenerative orthopedics. Mayo Clin Proc 88:1323

Fabani MM, Gait MJ (2008) miR-122 targeting with LNA/2′-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates. RNA 14:336

Fang J, Zhu YY, Smiley E, Bonadio J, Rouleau JP, Goldstein SA, McCauley LK, Davidson BL, Roessler BJ (1996) Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. Proc Natl Acad Sci U S A 93:5753

Fang L, Du WW, Yang W, Rutnam ZJ, Peng C, Li H, O'Malley YQ, Askeland RW, Sugg S, Liu M, Mehta T, Deng Z, Yang BB (2012) MiR-93 enhances angiogenesis and metastasis by targeting LATS2. Cell Cycle 11:4352–4365

Farrell RE (2006) RNA methodologies. Rev Cell Biol Mol Med. Wiley-VCH Verlag GmbH & Co. KGaA

Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, Capogrossi MC, Martelli F (2008) MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem 283:15878

Feichtinger X, Muschitz C, Heimel P, Baierl A, Fahrleitner-Pammer A, Redl H, Resch H, Geiger E, Skalicky S, Dormann R, Plachel F, Pietschmann P, Grillari J, Hackl M, Kocijan R (2018) Bone-related circulating MicroRNAs miR-29b-3p, miR-550a-3p, and miR-324-3p and their association to bone microstructure and histomorphometry. Sci Rep 8:4867

Feng B, Chen S, McArthur K, Wu Y, Sen S, Ding Q, Feldman RD, Chakrabarti S (2011) miR-146a-mediated extracellular matrix protein production in chronic diabetes complications. Diabetes 60:2975

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806

Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D (2008) miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell 15:272

Fitzgerald KA, Malhotra M, Curtin CM, O'Brien FJ, O'Driscoll CM (2015) Life in 3D is never flat: 3D models to optimise drug delivery. J Control Release 215:39–54

Fitzgerald KA, Guo J, Raftery RM, Castano IM, Curtin CM, Gooding M, Darcy R, O'Brien FJ, O'Driscoll CM (2016) Nanoparticle-mediated siRNA delivery assessed in a 3D co-culture model simulating prostate cancer bone metastasis. Int J Pharm 511:1058

Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW (2006) Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnol Lett 28:1601

Gabler J, Ruetze M, Kynast KL, Grossner T, Diederichs S, Richter W (2015) Stage-specific miRs in chondrocyte maturation: differentiation-dependent and hypertrophy-related miR clusters and the miR-181 family. Tissue Eng A 21:2840

Ghosh R, Singh LC, Shohet JM, Gunaratne PH (2012) A gold nanoparticle platform for the delivery of functional microRNAs into cancer cells. Biomaterials 34:807
Giger EV, Castagner B, Raikkonen J, Monkkonen J, Leroux JC (2013) siRNA transfection with calcium phosphate nanoparticles stabilized with PEGylated chelators. Adv Healthc Mater 2:134
Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, Bertone P, Caldas C (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 16:991
Gordeladze JO, Reseland JE, Duroux-Richard I, Apparailly F, Jorgensen C (2010) From stem cells to bone: phenotype acquisition, stabilization, and tissue engineering in animal models. ILAR J 51:42
Graham FL, van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456
GrandPré T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439
Gray KA, Yates B, Seal RL, Wright MW, Bruford EA (2015) Genenames.org: the HGNC resources in 2015. Nucleic Acids Res 43:D1079
Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34:D140
Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27:91
Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell 106:23
Grundmann S, Hans FP, Kinniry S, Heinke J, Helbing T, Bluhm F, Sluijter JP, Hoefer I, Pasterkamp G, Bode C, Moser M (2011) MicroRNA-100 regulates neovascularization by suppression of mammalian target of rapamycin in endothelial and vascular smooth muscle cells. Circulation 123:999
Grunhagen J, Ott CE (2013) On microRNA-214 suppressing osteogenic differentiation of C2C12 myoblast cells by targeting Osterix. Bone 57:325
Guk K, Lim H, Kim B, Hong M, Khang G, Lee D (2013) Acid-cleavable ketal containing poly(beta-amino ester) for enhanced siRNA delivery. Int J Pharm 453:541
Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466:835
Guo Z, Peng H, Kang J, Sun D (2016) Cell-penetrating peptides: possible transduction mechanisms and therapeutic applications. Biomed Rep 4:528
Gupta P, Authimoolam SP, Hilt JZ, Dziubla TD (2015) Quercetin conjugated poly(beta-amino esters) nanogels for the treatment of cellular oxidative stress. Acta Biomater 27:194
Hackl M, Jakobi T, Blom J, Doppmeier D, Brinkrolf K, Szczepanowski R, Bernhart SH, Honer Zu Siederdissen C, Bort JA, Wieser M, Kunert R, Jeffs S, Hofacker IL, Goesmann A, Puhler A, Borth N, Grillari J (2011) Next-generation sequencing of the Chinese hamster ovary microRNA transcriptome: identification, annotation and profiling of microRNAs as targets for cellular engineering. J Biotechnol 153:62
Hackl M, Heilmeier U, Weilner S, Grillari J (2016) Circulating microRNAs as novel biomarkers for bone diseases – complex signatures for multifactorial diseases? Mol Cell Endocrinol. 2015 The Authors, Ireland. Published by Elsevier Ireland Ltd. p 83
Haensler J, Szoka FC Jr (1993) Synthesis and characterization of a trigalactosylated bisacridine compound to target DNA to hepatocytes. Bioconjug Chem 4:85
Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950
Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404:293
Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293:1146
Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ (2008) MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A 105:1516

Hassan MQ, Maeda Y, Taipaleenmaki H, Zhang W, Jafferji M, Gordon JA, Li Z, Croce CM, Van Wijnen AJ, Stein JL, Stein GS, Lian JB (2012) miR-218 directs a Wnt signaling circuit to promote differentiation of osteoblasts and osteomimicry of metastatic cancer cells. J Biol Chem 287:42084–42092

Hatakeyama Y, Tuan RS, Shum L (2004) Distinct functions of BMP4 and GDF5 in the regulation of chondrogenesis. J Cell Biochem 91:1204

Hattori T, Müller C, Gebhard S, Bauer E, Pausch F, Schlund B, Bösl MR, Hess A, Surmann-Schmitt C, von der Mark H, de Crombrugghe B, von der Mark K (2010) SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. Development 137:901

Haussecker D (2014) Current issues of RNAi therapeutics delivery and development. J Control Release 195:49

Hedberg EL, Shih CK, Lemoine JJ, Timmer MD, Liebschner MA, Jansen JA, Mikos AG (2005) In vitro degradation of porous poly(propylene fumarate)/poly(DL-lactic-co-glycolic acid) composite scaffolds. Biomaterials 26:3215

Hoseinzadeh S, Atashi A, Soleimani M, Alizadeh E, Zarghami N (2016) MiR-221-inhibited adipose tissue-derived mesenchymal stem cells bioengineered in a nano-hydroxy apatite scaffold. In Vitro Cell Dev Biol Anim 52:479

Hu R, Li H, Liu W, Yang L, Tan Y, Luo X (2010) Targeting miRNAs in osteoblast differentiation and bone formation. Expert Opin Ther Targets 14:1109

Huang J, Zhao L, Xing L, Chen D (2010) MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. Stem Cells 28:357

Huey DJ, Hu JC, Athanasiou KA (2012) Unlike bone, cartilage regeneration remains elusive. Science (New York, NY) 338:917

Huh YH, Ryu J-H, Chun J-S (2007) Regulation of type II collagen expression by histone deacetylase in articular chondrocytes. J Biol Chem 282:17123

Hutvagner G, Zamore PDA (2002) microRNA in a multiple-turnover RNAi enzyme complex. Science 297:2056

Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293:834

Huynh CT, Nguyen MK, Naris M, Tonga GY, Rotello VM, Alsberg E (2016) Light-triggered RNA release and induction of hMSC osteogenesis via photodegradable, dual-crosslinked hydrogels. Nanomedicine (Lond) 11:1535

Hwang DW, Son S, Jang J, Youn H, Lee S, Lee D, Lee YS, Jeong JM, Kim WJ, Lee DS (2011) A brain-targeted rabies virus glycoprotein-disulfide linked PEI nanocarrier for delivery of neurogenic microRNA. Biomaterials 32:4968

Icli B, Wara AK, Moslehi J, Sun X, Plovie E, Cahill M, Marchini JF, Schissler A, Padera RF, Shi J, Cheng HW, Raghuram S, Arany Z, Liao R, Croce K, MacRae C, Feinberg MW (2013) MicroRNA-26a regulates pathological and physiological angiogenesis by targeting BMP/SMAD1 signaling. Circ Res 113:1231

Ilina P, Hyvonen Z, Saura M, Sandvig K, Yliperttula M, Ruponen M (2012) Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems. J Control Release 163:385

Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A (2008) Integrative MicroRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. PLoS One 3:e3740

ISBLab (2013) miRTarBase: the experimentally validated microRNA-target interactions database. In: National Chiao Tung University (ed) Department of Biological Science and Technology I.o. B.a.S.B., miRTarBase Release 45. Taiwan © ISBLab, Hsinchu

Ishizuka A, Siomi MC, Siomi HA (2002) Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev 16:2497

Itoh T, Nozawa Y, Akao Y (2009) MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. J Biol Chem 284:19272

Jacobsen A, Silber J, Harinath G, Huse JT, Schultz N, Sander C (2013) Analysis of microRNA-target interactions across diverse cancer types. Nat Struct Mol Biol 20:1325

Jaklenec A, Stamp A, Deweerd E, Sherwin A, Langer R (2012) Progress in the tissue engineering and stem cell industry "are we there yet?". Tissue Eng B-Rev 18:155

James EN, Delany AM, Nair LS (2014) Post-transcriptional regulation in osteoblasts using localized delivery of miR-29a inhibitor from nanofibers to enhance extracellular matrix deposition. Acta Biomater 10:3571

Janssen HL, Kauppinen S, Hodges MR (2013) HCV infection and miravirsen. N Engl J Med 369:878

Jee YH, Wang J, Yue S, Jennings M, Clokie SJ, Nilsson O, Lui JC, Baron J (2018) mir-374-5p, mir-379-5p, and mir-503-5p regulate proliferation and hypertrophic differentiation of growth plate chondrocytes in male rats. Endocrinology 159:1469

Jensen ED, Gopalakrishnan R, Westendorf JJ (2010) Regulation of gene expression in osteoblasts. Biofactors 36:25

Jere D, Xu CX, Arote R, Yun CH, Cho MH, Cho CS (2008) Poly(beta-amino ester) as a carrier for si/shRNA delivery in lung cancer cells. Biomaterials 29:2535

Johnson EE, Urist MR (1998) One-stage lengthening of femoral nonunion augmented with human bone morphogenetic protein. Clin Orthop Relat Res, 347:105

Johnston RJ, Hobert O (2003) A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. Nature 426:845

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265

Jones E, Mazirka P, McNurlan MA, Brink P, Caso G (2017) Inhibition of human prostate cancer growth by mesenchymal stem cells delivering MiR-16. FASEB J 31:lb177

Kapinas K, Kessler CB, Delany AM (2009) miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signaling. J Cell Biochem 108:216

Karlsen TA, Jakobsen RB, Mikkelsen TS, Brinchmann JE (2013) microRNA-140 targets RALA and regulates chondrogenic differentiation of human mesenchymal stem cells by translational enhancement of SOX9 and ACAN. Stem Cells Dev 23:290

Karlsen TA, de Souza GA, Ødegaard B, Engebretsen L, Brinchmann JE (2016) microRNA-140 inhibits inflammation and stimulates chondrogenesis in a model of interleukin 1β-induced osteoarthritis. Mol Ther Nucleic Acids 5:e373

Ketting RF, Haverkamp TH, van Luenen HG, Plasterk RH (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell 99:133

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev 15:2654

Khademhosseini A, Vacanti JP, Langer R (2009) Progress in tissue engineering. Sci Am 300:64

Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209

Kim KM, Lim S-K (2014) Role of miRNAs in bone and their potential as therapeutic targets. Curr Opin Pharmacol 16:133

Kim YJ, Bae SW, Yu SS, Bae YC, Jung JS (2009) miR-196a regulates proliferation and osteogenic differentiation in mesenchymal stem cells derived from human adipose tissue. J Bone Miner Res Off J Am Soc Bone Miner Res 24:816

Kim NH, Choi SH, Kim CH, Lee CH, Lee TR, Lee AY (2014) Reduced MiR-675 in exosome in H19 RNA-related melanogenesis via MITF as a direct target. J Invest Dermatol 134:1075

Kimelman-Bleich N, Pelled G, Zilberman Y, Kallai I, Mizrahi O, Tawackoli W, Gazit Z, Gazit D (2011) Targeted gene-and-host progenitor cell therapy for nonunion bone fracture repair. Mol Ther: J Am Soc Gene Ther 19:53

Knight SW, Bass BL (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. Science 293:2269

Kozarewa I, Ning Z, Quail MA, Sanders MJ, Berriman M, Turner DJ (2009) Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Nat Methods 6:291

Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M (2005) Silencing of microRNAs in vivo with 'antagomirs'. Nature 438:685

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294:853

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 294:858

Laufs S, Guenechea G, Gonzalez-Murillo A, Zsuzsanna Nagy K, Luz Lozano M, del Val C, Jonnakuty S, Hotz-Wagenblatt A, Jens Zeller W, Bueren JA, Fruehauf S (2006) Lentiviral vector integration sites in human NOD/SCID repopulating cells. J Gene Med 8:1197

Leblebici B, Adam M, Bagis S, Tarim AM, Noyan T, Akman MN, Haberal MA (2006) Quality of life after burn injury: the impact of joint contracture. J Burn Care Res Engl 27:864

Lee RC, Ambros V (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294:862

Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843

Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 21:4663

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425:415

Lee K, Silva EA, Mooney DJ (2011) Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. J R Soc Interface 8:153

Lee K, Oh MH, Lee MS, Nam YS, Park TG, Jeong JH (2013) Stabilized calcium phosphate nano-aggregates using a dopa-chitosan conjugate for gene delivery. Int J Pharm 445:196

Lee MS, Lee JE, Byun E, Kim NW, Lee K, Lee H, Sim SJ, Lee DS, Jeong JH (2014) Target-specific delivery of siRNA by stabilized calcium phosphate nanoparticles using dopa–hyaluronic acid conjugate. J Control Release 192:122–130

Lefebvre V, Dumitriu B, Penzo-Méndez A, Han Y, Pallavi B (2007) Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. Int J Biochem Cell Biol 39:2195

Legendre F, Ollitrault D, Gomez-Leduc T, Bouyoucef M, Hervieu M, Gruchy N, Mallein-Gerin F, Leclercq S, Demoor M, Galéra P (2017) Enhanced chondrogenesis of bone marrow-derived stem cells by using a combinatory cell therapy strategy with BMP-2/TGF-β1, hypoxia, and COL1A1/HtrA1 siRNAs. Sci Rep 7:3406

Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, Candi E (2008) miR-203 represses 'stemness' by repressing DeltaNp63. Cell Death Differ 15:1187

Lepper C, Partridge TA, Fan CM (2011) An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Development 138:3639

Levy O, Ruvinov E, Reem T, Granot Y, Cohen S (2010) Highly efficient osteogenic differentiation of human mesenchymal stem cells by eradication of STAT3 signaling. Int J Biochem Cell Biol 42:1823

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15

Li H, Li WX, Ding SW (2002) Induction and suppression of RNA silencing by an animal virus. Science 296:1319

Li Z, Hassan MQ, Volinia S, van Wijnen AJ, Stein JL, Croce CM, Lian JB, Stein GS (2008) A microRNA signature for a BMP2-induced osteoblast lineage commitment program. Proc Natl Acad Sci U S A 105:13906

Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2009a) Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 284:15676

Li H, Xie H, Liu W, Hu R, Huang B, Tan YF, Xu K, Sheng ZF, Zhou HD, Wu XP, Luo XH (2009b) A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. J Clin Invest 119:3666

Li J, Chen YC, Tseng YC, Huang L (2010) Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery. J Control Release 142:416

Li Y, Fan L, Liu S, Liu W, Zhang H, Zhou T, Wu D, Yang P, Shen L, Chen J, Jin Y (2013) The promotion of bone regeneration through positive regulation of angiogenic-osteogenic coupling using microRNA-26a. Biomaterials 34:5048

Li J, Yu X, Wang Y, Yuan Y, Xiao H, Cheng D, Shuai X (2014) A reduction and pH dual-sensitive polymeric vector for long-circulating and tumor-targeted siRNA delivery. Adv Mater 26:8217

Li KC, Lo SC, Sung LY, Liao YH, Chang YH, Hu YC (2016) Improved calvarial bone repair by hASCs engineered with Cre/loxP-based baculovirus conferring prolonged BMP-2 and MiR-148b co-expression. J Tissue Eng Regen Med 11:3068

Li KC, Chang YH, Yeh CL, Hu YC (2016a) Healing of osteoporotic bone defects by baculovirus-engineered bone marrow-derived MSCs expressing MicroRNA sponges. Biomaterials 74:155

Li Y, Dal-Pra S, Mirotsou M, Jayawardena TM, Hodgkinson CP, Bursac N, Dzau VJ (2016b) Tissue-engineered 3-dimensional (3D) microenvironment enhances the direct reprogramming of fibroblasts into cardiomyocytes by microRNAs. Sci Rep 6:38815

Liao XB, Zhang ZY, Yuan K, Liu Y, Feng X, Cui RR, Hu YR, Yuan ZS, Gu L, Li SJ, Mao DA, Lu Q, Zhou XM, de Jesus Perez VA, Yuan LQ (2013a) MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells. Endocrinology 154:3344

Liao L, Yang X, Su X, Hu C, Zhu X, Yang N, Chen X, Shi S, Shi S, Jin Y (2013b) Redundant miR-3077-5p and miR-705 mediate the shift of mesenchymal stem cell lineage commitment to adipocyte in osteoporosis bone marrow. Cell Death Dis 4:e600

Liao Y-H, Chang Y-H, Sung L-Y, Li K-C, Yeh C-L, Yen T-C, Hwang S-M, Lin K-J, Hua Y-C (2014a) Osteogenic differentiation of adipose-derived stem cells and calvarial defect repair using baculovirus-mediated co-expression of BMP-2 and miR-148b. Biomaterials 35:4901

Liao YH, Chang YH, Sung LY, Li KC, Yeh CL, Yen TC, Hwang SM, Lin KJ, Hu YC (2014b) Osteogenic differentiation of adipose-derived stem cells and calvarial defect repair using baculovirus-mediated co-expression of BMP-2 and miR-148b. Biomaterials 35:4901

Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP (2003) The microRNAs of *Caenorhabditis elegans*. Genes Dev 17:991

Lin RC, Weeks KL, Gao XM, Williams RB, Bernardo BC, Kiriazis H, Matthews VB, Woodcock EA, Bouwman RD, Mollica JP, Speirs HJ, Dawes IW, Daly RJ, Shioi T, Izumo S, Febbraio MA, Du XJ, McMullen JR (2010) PI3K (p110 alpha) protects against myocardial infarction-induced heart failure: identification of PI3K-regulated miRNA and mRNA. Arterioscler Thromb Vasc Biol 30:724

Lingel A, Simon B, Izaurralde E, Sattler M (2003) Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. Nature 426:465

Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, Johnson JM, Cummins JM, Raymond CK, Dai H, Chau N, Cleary M, Jackson AL, Carleton M, Lim L (2007) Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol 27:2240

Liu YP, Berkhout B (1809) miRNA cassettes in viral vectors: problems and solutions. Biochim Biophys Acta (BBA) – Gene Regul Mech 732:2011

Liu C-F, Lefebvre V (2015) The transcription factors SOX9 and SOX5/SOX6 cooperate genome-wide through super-enhancers to drive chondrogenesis. Nucleic Acids Res 43:8183

Liu L, Shu S, Cheung GS, Wei X (2016) Effect of miR-146a/bFGF/PEG-PEI nanoparticles on inflammation response and tissue regeneration of human dental pulp cells. Biomed Res Int 2016:3892685

Lolli A, Lambertini E, Penolazzi L, Angelozzi M, Morganti C, Franceschetti T, Pelucchi S, Gambari R, Piva R (2014) Pro-chondrogenic effect of miR-221 and slug depletion in human MSCs. Stem Cell Rev 10:841

Lolli A, Narcisi R, Lambertini E, Penolazzi L, Angelozzi M, Kops N, Gasparini S, van Osch GJ, Piva R (2016) Silencing of Antichondrogenic MicroRNA-221 in human mesenchymal stem cells promotes cartilage repair in vivo. Stem Cells 34:1801

Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U (2004) Nuclear export of microRNA precursors. Science 303:95

Luzi E, Marini F, Sala SC, Tognarini I, Galli G, Brandi ML (2008) Osteogenic differentiation of human adipose tissue-derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. J Bone Miner Res Off J Am Soc Bone Miner Res 23:287

Lytle JR, Yario TA, Steitz JA (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc Natl Acad Sci 104:9667

Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, Teruya-Feldstein J, Reinhardt F, Onder TT, Valastyan S, Westermann F, Speleman F, Vandesompele J, Weinberg RA (2010) miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nat Cell Biol 12:247

MacLaughlin FC, Mumper RJ, Wang J, Tagliaferri JM, Gill I, Hinchcliffe M, Rolland AP (1998) Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J Control Release 56:259

Madhyastha R, Madhyastha H, Nakajima Y, Omura S, Maruyama M (2012) MicroRNA signature in diabetic wound healing: promotive role of miR-21 in fibroblast migration. Int Wound J 9:355

Mahboudi H, Soleimani M, Enderami SE, Kehtari M, Ardeshirylajimi A, Eftekhary M, Kazemi B (2018) Enhanced chondrogenesis differentiation of human induced pluripotent stem cells by MicroRNA-140 and transforming growth factor beta 3 (TGFβ3). Biologicals 52:30–36

Makeyev EV, Zhang J, Carrasco MA, Maniatis T (2007) The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol Cell 27:435

Malmo J, Sorgard H, Varum KM, Strand SP (2012) siRNA delivery with chitosan nanoparticles: molecular properties favoring efficient gene silencing. J Control Release 158:261

Mariner PD, Johannesen E, Anseth KS (2011) Manipulation of miRNA activity accelerates osteogenic differentiation of hMSCs in engineered 3D scaffolds. J Tissue Eng Regen Med 6:314

Mariner PD, Johannesen E, Anseth KS (2012) Manipulation of miRNA activity accelerates osteogenic differentiation of hMSCs in engineered 3D scaffolds. J Tissue Eng Regen Med 6:314

Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, andTuschl T (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110:563

Martini FH, Nath JL, Bartholomew EF (2006) Fundamentals of anatomy and physiology, 9th edn. P.E.I. Editorial, San Francisco

McArthur K, Feng B, Wu Y, Chen S, Chakrabarti S (2011) MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. Diabetes 60:1314

McCarthy JJ (2008) MicroRNA-206: the skeletal muscle-specific myomiR. Biochim Biophys Acta 1779:682

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805

McKiernan P, Cunningham O, Greene C, Cryan S-A (2013) Targeting miRNA-based medicines to cystic fibrosis airway epithelial cells using nanotechnology. Int J Nanomedicine 8:3907

Mencia Castano I, Curtin CM, Shaw G, Murphy JM, Duffy GP, O'Brien FJ (2015) A novel collagen-nanohydroxyapatite microRNA-activated scaffold for tissue engineering applications

capable of efficient delivery of both miR-mimics and antagomiRs to human mesenchymal stem cells. J Control Release 200:42
Mencia Castano I, Curtin CM, Duffy GP, O'Brien FJ (2016) Next generation bone tissue engineering: non-viral miR-133a inhibition using collagen-nanohydroxyapatite scaffolds rapidly enhances osteogenesis. Sci Rep 6:27941
Mencia Castano I, Curtin CM, Duffy GP, O'Brien FJ (2018) Harnessing a novel role of miR-16 in osteogenesis for scaffold-based bone tissue engineering. Tissue Eng A 25:24
Mercado AT, Yeh JM, Chin TY, Chen WS, Chen-Yang YW, Chen CY (2016) The effect of chemically modified electrospun silica nanofiber on the mRNA and miRNA expression profile of neural stem cell differentiation. J Biomed Mater Res A 104:2730
Mercken EM, Majounie E, Ding J, Guo R, Kim J, Bernier M, Mattison J, Cookson MR, Gorospe M, de Cabo R, Abdelmohsen K (2013) Age-associated miRNA alterations in skeletal muscle from rhesus monkeys reversed by caloric restriction. Aging (Albany NY) 5:692
Midgley AC, Bowen T, Phillips AO, Steadman R (2014) MicroRNA-7 inhibition rescues age-associated loss of epidermal growth factor receptor and hyaluronan-dependent differentiation in fibroblasts. Aging Cell 13:235
Midoux P, Pichon C, Yaouanc JJ, Jaffres PA (2009) Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carrier. Br J Pharmacol 157:166
Miller KJ, Brown DA, Ibrahim MM, Ramchal TD, Levinson H (2015) MicroRNAs in skin tissue engineering. Adv Drug Deliv Rev 88:16
miRBase (2013) ed: Faculty of Life Sciences University of Manchester
Mizuno Y, Yagi K, Tokuzawa Y, Kanesaki-Yatsuka Y, Suda T, Katagiri T, Fukuda T, Maruyama M, Okuda A, Amemiya T, Kondoh Y, Tashiro H, Okazaki Y (2008) miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. Biochem Biophys Res Commun 368:267
Mizuno Y, Tokuzawa Y, Ninomiya Y, Yagi K, Yatsuka-Kanesaki Y, Suda T, Fukuda T, Katagiri T, Kondoh Y, Amemiya T, Tashiro H, Okazaki Y (2009) miR-210 promotes osteoblastic differentiation through inhibition of AcvR1b. FEBS Lett 583:2263
Monaghan M, Pandit A (2011) RNA interference therapy via functionalized scaffolds. Adv Drug Deliv Rev 63:197
Monaghan M, Browne S, Schenke-Layland K, Pandit A (2014) A collagen-based scaffold delivering exogenous microrna-29B to modulate extracellular matrix remodeling. Mol Ther: J Am Soc Gene Ther 22:786
Monaghan MG, Holeiter M, Brauchle E, Layland SL, Lu Y, Deb A, Pandit A, Nsair A, Schenke-Layland K (2018) Exogenous miR-29B delivery through a hyaluronan-based injectable system yields functional maintenance of the infarcted myocardium. Tissue Eng Part A 24:57
Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicenti F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C, von Kalle C, Naldini L (2009) The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. J Clin Invest 119:964
Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev 16:720
Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 101:533
Mowa MB, Crowther C, Arbuthnot P (2010) Therapeutic potential of adenoviral vectors for delivery of expressed RNAi activators. Expert Opin Drug Deliv 7:1373
Nakasa T, Ishikawa M, Shi M, Shibuya H, Adachi N, Ochi M (2010) Acceleration of muscle regeneration by local injection of muscle-specific microRNAs in rat skeletal muscle injury model. J Cell Mol Med 14:2495

Nelson CE, Kim AJ, Adolph EJ, Gupta MK, Yu F, Hocking KM, Davidson JM, Guelcher SA, Duvall CL (2014) Tunable delivery of siRNA from a biodegradable scaffold to promote angiogenesis in vivo. Adv Mater 26:607

Nerem RM (1991) Cellular engineering. Ann Biomed Eng 19:529

Nguyen MK, Jeon O, Krebs MD, Schapira D, Alsberg E (2014) Sustained localized presentation of RNA interfering molecules from in situ forming hydrogels to guide stem cell osteogenic differentiation. Biomaterials 35:6278

Nguyen LH, Diao HJ, Chew SY (2015) MicroRNAs and their potential therapeutic applications in neural tissue engineering. Adv Drug Deliv Rev 88:53

Nguyen LH, Gao M, Lin J, Wu W, Wang J, Chew SY (2017) Three-dimensional aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to direct axon regeneration in spinal cord injury treatment. Sci Rep 7:42212

Niemeyer P, Pestka JM, Kreuz PC, Erggelet C, Schmal H, Suedkamp NP, Steinwachs M (2008) Characteristic complications after autologous chondrocyte implantation for cartilage defects of the knee joint. Am J Sports Med 36:2091

Nunes DN, Dias-Neto E, Cardo-Vila M, Edwards JK, Dobroff AS, Giordano RJ, Mandelin J, Brentani HP, Hasselgren C, Yao VJ, Marchio S, Pereira CA, Passetti F, Calin GA, Sidman RL, Arap W, Pasqualini R (2015) Synchronous down-modulation of miR-17 family members is an early causative event in the retinal angiogenic switch. Proc Natl Acad Sci U S A 112:3770

Nykanen A, Haley B, Zamore PD (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107:309

Obernosterer G, Martinez J, Alenius M (2007) Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. Nat Protoc 2:1508

O'Brien FJ, Harley BA, Waller MA, Yannas IV, Gibson LJ, Prendergast PJ (2007) The effect of pore size on permeability and cell attachment in collagen scaffolds for tissue engineering. Technol Health Care 15:3

Ohler U, Yekta S, Lim LP, Bartel DP, Burge CB (2004) Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification. RNA 10:1309

Okamura K, Liu N, Lai EC (2009) Distinct mechanisms for microRNA strand selection by Drosophila Argonautes. Mol Cell 36:431

Ørom UA, Lund AH (2010) Experimental identification of microRNA targets. Gene 451:1

Ozsolak F, Milos PM (2011) RNA sequencing: advances, challenges and opportunities. Nat Rev Genet 12:87

Pack DW, Hoffman AS, Pun S, Stayton PS (2005) Design and development of polymers for gene delivery. Nat Rev Drug Discov 4:581

Pan Y, Zhang Y, Jia T, Zhang K, Li J, Wang L (2012a) Development of a microRNA delivery system based on bacteriophage MS2 virus-like particles. FEBS J 279:1198

Pan Y, Jia T, Zhang Y, Zhang K, Zhang R, Li J, Wang L (2012b) MS2 VLP-based delivery of microRNA-146a inhibits autoantibody production in lupus-prone mice. Int J Nanomedicine 7:5957

Park K, Lee K-M, Yoon D, Park K, Choi W, Lee J, Kim S-H (2016) Inhibition of microRNA-449a prevents IL-1β-induced cartilage destruction via SIRT1. Osteoarthr Cartil 24:2153

Pascual-Gil S, Garbayo E, Díaz-Herráez P, Prosper F, Blanco-Prieto MJ (2015) Heart regeneration after myocardial infarction using synthetic biomaterials. J Control Release 203:23

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408:86

Pastar I, Khan AA, Stojadinovic O, Lebrun EA, Medina MC, Brem H, Kirsner RS, Jimenez JJ, Leslie C, Tomic-Canic M (2012) Induction of specific microRNAs inhibits cutaneous wound healing. J Biol Chem 287:29324

Pearson H (2006) Genetics: what is a gene? Nature 441:398

Pedraza CE, Bassett DC, McKee MD, Nelea V, Gbureck U, Barralet JE (2008) The importance of particle size and DNA condensation salt for calcium phosphate nanoparticle transfection. Biomaterials 29:3384

Pekarsky Y, Balatti V, Croce CM (2018) BCL2 and miR-15/16: from gene discovery to treatment. Cell Death Differ 25:21

Pelled G, Ben-Arav A, Hock C, Reynolds DG, Yazici C, Zilberman Y, Gazit Z, Awad H, Gazit D, Schwarz EM (2010) Direct gene therapy for bone regeneration: gene delivery, animal models, and outcome measures. Tissue Eng Part B Rev 16:13

Pena JT, Sohn-Lee C, Rouhanifard SH, Ludwig J, Hafner M, Mihailovic A, Lim C, Holoch D, Berninger P, Zavolan M, Tuschl T (2009) miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. Nat Methods 6:139

Peng WJ, Tao JH, Mei B, Chen B, Li BZ, Yang GJ, Zhang Q, Yao H, Wang BX, He Q, Wang J (2012) MicroRNA-29: a potential therapeutic target for systemic sclerosis. Expert Opin Ther Targets 16:875

Perruisseau-Carrier C, Jurga M, Forraz N, McGuckin CP (2011) miRNAs stem cell reprogramming for neuronal induction and differentiation. Mol Neurobiol 43:215

Petersen M, Wengel J (2003) LNA: a versatile tool for therapeutics and genomics. Trends Biotechnol 21:74

Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, Mercatanti A, Hammond S, Rainaldi G (2006) MicroRNAs modulate the angiogenic properties of HUVECs. Blood 108:3068

Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW, van Rooij E, Olson EN (2011) MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. Circ Res 109:670

Qian X, Long L, Shi Z, Liu C, Qiu M, Sheng J, Pu P, Yuan X, Ren Y, Kang C (2014) Star-branched amphiphilic PLA-b-PDMAEMA copolymers for co-delivery of miR-21 inhibitor and doxorubicin to treat glioma. Biomaterials 35:2322

Qureshi AT, Monroe WT, Dasa V, Gimble JM, Hayes DJ (2013) miR-148b eNanoparticle conjugates for light mediated osteogenesis of human adipose stromal/stem cells. Biomaterials 34:7799

Qureshi AT, Doyle A, Chen C, Coulon D, Dasa V, Del Piero F, Levi B, Monroe WT, Gimble JM, Hayes DJ (2015) Photoactivated miR-148b-nanoparticle conjugates improve closure of critical size mouse calvarial defects. Acta Biomater 12:166

Raftery RM, Walsh DP, Mencía Castaño I, Heise A, Duffy GP, Cryan S-A, O'Brien FJ (2016) Delivering nucleic-acid based nanomedicines on biomaterial scaffolds for orthopedic tissue repair: challenges, progress and future perspectives. Adv Mater n/a

Reddy KJ, Gilman M (2001) Preparation of bacterial RNA. Curr Protoc Mol Biol. Wiley

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 403:901

Remaut K, Symens N, Lucas B, Demeester J, De Smedt SC (2010) Efficient delivery of intact phosphodiester oligonucleotides by poly-beta-amino esters. J Control Release 144:65

Rinn JL, Chang HY (2012) Genome regulation by long noncoding RNAs. Annu Rev Biochem 81:145

Sakurai H, Kawabata K, Sakurai F, Nakagawa S, Mizuguchi H (2008) Innate immune response induced by gene delivery vectors. Int J Pharm 354:9

Santos JL, Pandita D, Rodrigues J, Pego AP, Granja PL, Tomas H (2011) Non-viral gene delivery to mesenchymal stem cells: methods, strategies and application in bone tissue engineering and regeneration. Curr Gene Ther 11:46

Santos-Carballal B, Aaldering LJ, Ritzefeld M, Pereira S, Sewald N, Moerschbacher BM, Gotte M, Goycoolea FM (2015) Physicochemical and biological characterization of chitosan-microRNA nanocomplexes for gene delivery to MCF-7 breast cancer cells. Sci Rep 5:13567

Sathy BN, Olvera D, Gonzalez-Fernandez T, Cunniffe GM, Pentlavalli S, Chambers P, Jeon O, Alsberg E, McCarthy HO, Dunne N, Haut Donahue TL, Kelly DJ (2017) RALA complexed α-TCP nanoparticle delivery to mesenchymal stem cells induces bone formation in tissue engineered constructs in vitro and in vivo. J Mater Chem B 5:1753

Schade A, Delyagina E, Scharfenberg D, Skorska A, Lux C, David R, Steinhoff G (2013) Innovative strategy for microRNA delivery in human mesenchymal stem cells via magnetic nanoparticles. Int J Mol Sci 14:10710

Schade A, Muller P, Delyagina E, Voronina N, Skorska A, Lux C, Steinhoff G, David R (2014) Magnetic nanoparticle based nonviral microRNA delivery into freshly isolated CD105(+) hMSCs. Stem Cells Int 2014:197154

Schirle NT, Sheu-Gruttadauria J, MacRae IJ (2014) Structural basis for microRNA targeting. Science 346:608

Schmidt CE, Leach JB (2003) Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng 5:293

Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, Schlegel J (2002) Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthr Cartil 10:62

Schneider MR, Samborski A, Bauersachs S, Zouboulis CC (2013) Differentially regulated microRNAs during human sebaceous lipogenesis. J Dermatol Sci 70:88

Schoolmeesters A, Eklund T, Leake D, Vermeulen A, Smith Q, Force Aldred S, Fedorov Y (2009) Functional profiling reveals critical role for miRNA in differentiation of human mesenchymal stem cells. PLoS One 4:e5605

Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115:199

Shevchenko RV, James SL, James SE (2010) A review of tissue-engineered skin bioconstructs available for skin reconstruction. J R Soc Interface 7:229

Shi S, Han L, Deng L, Zhang Y, Shen H, Gong T, Zhang Z, Sun X (2014) Dual drugs (microRNA-34a and paclitaxel)-loaded functional solid lipid nanoparticles for synergistic cancer cell suppression. J Control Release 194:228

Shoji T, Nakasa T, Yamasaki K, Kodama A, Miyaki S, Niimoto T, Okuhara A, Kamei N, Adachi N, Ochi M (2012) The effect of intra-articular injection of MicroRNA-210 on ligament healing in a rat model. Am J Sports Med 40:2470

Silahtaroglu AN, Nolting D, Dyrskjøt L, Berezikov E, Møller M, Tommerup N, Kauppinen S (2007) Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification. Nat Protoc 2:2520

Sinha M, Ghatak S, Roy S, Sen CK (2015) microRNA-200b as a switch for inducible adult angiogenesis. Antioxid Redox Signal 22:1257

Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G (2000) The lin-41 RBCC gene acts in the *C. elegans* heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell 5:659

Sluijter JP, van Mil A, van Vliet P, Metz CH, Liu J, Doevendans PA, Goumans MJ (2010) MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arterioscler Thromb Vasc Biol 30:859

Sriram M, Sainitya R, Kalyanaraman V, Dhivya S, Selvamurugan N (2015) Biomaterials mediated microRNA delivery for bone tissue engineering. Int J Biol Macromol 74:404

Stappert L, Borghese L, Roese-Koerner B, Weinhold S, Koch P, Terstegge S, Uhrberg M, Wernet P, Brustle O (2013) MicroRNA-based promotion of human neuronal differentiation and subtype specification. PLoS One 8:e59011

Stappert L, Roese-Koerner B, Brüstle O (2015) The role of microRNAs in human neural stem cells, neuronal differentiation and subtype specification. Cell Tissue Res 359:47

Stenberg J, de Windt TS, Synnergren J, Hynsjö L, van der Lee J, Saris DB, Brittberg M, Peterson L, Lindahl A (2014) Clinical outcome 3 years after autologous chondrocyte implantation does not correlate with the expression of a predefined gene marker set in chondrocytes prior to

implantation but is associated with critical signaling pathways. Orthop J Sports Med 2:2325967114550781
Stevanato L, Sinden JD (2014) The effects of microRNAs on human neural stem cell differentiation in two- and three-dimensional cultures. Stem Cell Res Ther 5:49
Stevens MM, George JH (2005) Exploring and engineering the cell surface interface. Science 310:1135
Suarez Y, Sessa WC (2009) MicroRNAs as novel regulators of angiogenesis. Circ Res 104:442
Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC (2007) Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ Res 100:1164
Suh EJ, Remillard MY, Legesse-Miller A, Johnson EL, Lemons JM, Chapman TR, Forman JJ, Kojima M, Silberman ES, Coller HA (2012) A microRNA network regulates proliferative timing and extracellular matrix synthesis during cellular quiescence in fibroblasts. Genome Biol 13:R121
Sun Q, Zhang Y, Yang G, Chen X, Cao G, Wang J, Sun Y, Zhang P, Fan M, Shao N, Yang X (2008) Transforming growth factor-beta-regulated miR-24 promotes skeletal muscle differentiation. Nucleic Acids Res 36:2690
Sun Q, Mao S, Li H, Zen K, Zhang CY, Li L (2013) Role of miR-17 family in the negative feedback loop of bone morphogenetic protein signaling in neuron. PLoS One 8:e83067
Sundaram GM, Common JE, Gopal FE, Srikanta S, Lakshman K, Lunny DP, Lim TC, Tanavde V, Lane EB, Sampath P (2013) 'See-saw' expression of microRNA-198 and FSTL1 from a single transcript in wound healing. Nature 495:103
Sung LY, Chen CL, Lin SY, Hwang SM, Lu CH, Li KC, Lan AS, Hu YC (2013) Enhanced and prolonged baculovirus-mediated expression by incorporating recombinase system and in cis elements: a comparative study. Nucleic Acids Res 41:e139
Svaren J (2014) MicroRNA and transcriptional crosstalk in myelinating glia. Neurochem Int 77:50
Takeda K (2009) Delivery of magic bullets: on the still rocky road to gene therapy. Br J Pharmacol 157:151
Tan J, Yang L, Liu C, Yan Z (2017) MicroRNA-26a targets MAPK6 to inhibit smooth muscle cell proliferation and vein graft neointimal hyperplasia. Sci Rep 7:46602
TargetScan Release 6.2 (2012) In: Computing B.a. R., ed. © 2006–2012 Whitehead Institute for Biomedical Research, Cambridge, MA
Tay YM, Tam WL, Ang YS, Gaughwin PM, Yang H, Wang W, Liu R, George J, Ng HH, Perera RJ, Lufkin T, Rigoutsos I, Thomson AM, Lim B (2008a) MicroRNA-134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1. Stem Cells 26:17
Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I (2008b) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455:1124
Tayalia P, Mooney DJ (2009) Controlled growth factor delivery for tissue engineering. Adv Mater 21:3269
Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Snitow M, Morley M, Li D, Petrenko N, Zhou S, Lu M, Gao E, Koch WJ, Stewart KM, Morrisey EE (2015) A microRNA-hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. Sci Transl Med 7:279ra38
Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E (2004) Defining the epithelial stem cell niche in skin. Science 303:359
Ulrich-Vinther M, Maloney MD, Schwarz EM, Rosier R, O'keefe RJ (2003) Articular cartilage biology. J Am Acad Orthop Surg 11:421
van Amerongen MJ, Engel FB (2008) Features of cardiomyocyte proliferation and its potential for cardiac regeneration. J Cell Mol Med 12:2233
van Rooij E (2011) The art of microRNA research. Circ Res 108:219
van Rooij E (2012) Introduction to the series on microRNAs in the cardiovascular system. Circ Res 110:481
van Rooij E, Kauppinen S (2014) Development of microRNA therapeutics is coming of age. EMBO Mol Med 6:851

van Rooij E, Purcell AL, Levin AA (2012) Developing microRNA therapeutics. Circ Res 110:496
Várallyay E, Burgyán J, Havelda Z (2008) MicroRNA detection by northern blotting using locked nucleic acid probes. Nat Protoc 3:190
Varkonyi-Gasic E, Hellens RP (2011) Quantitative stem-loop RT-PCR for detection of microRNAs. Methods Mol Biol 744:145
Vimalraj S, Saravanan S, Vairamani M, Gopalakrishnan C, Sastry TP, Selvamurugan N (2016) A combinatorial effect of carboxymethyl cellulose based scaffold and microRNA-15b on osteoblast differentiation. Int J Biol Macromol 93:1457
Vo TN, Kasper FK, Mikos AG (2012) Strategies for controlled delivery of growth factors and cells for bone regeneration. Adv Drug Deliv Rev 64:1292
Vuorimaa E, Ketola TM, Green JJ, Hanzlikova M, Lemmetyinen H, Langer R, Anderson DG, Urtti A, Yliperttula M (2011) Poly(beta-amino ester)-DNA complexes: time-resolved fluorescence and cellular transfection studies. J Control Release 154:171
Wada S, Kato Y, Okutsu M, Miyaki S, Suzuki K, Yan Z, Schiaffino S, Asahara H, Ushida T, Akimoto T (2011) Translational suppression of atrophic regulators by microRNA-23a integrates resistance to skeletal muscle atrophy. J Biol Chem 286:38456
Wang S, Olson EN (2009) AngiomiRs – key regulators of angiogenesis. Curr Opin Genet Dev 19:205
Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN (2008) The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 15:261
Wang D, Zhang Z, O'Loughlin E, Lee T, Houel S, O'Carroll D, Tarakhovsky A, Ahn NG, Yi R (2012) Quantitative functions of Argonaute proteins in mammalian development. Genes Dev 26:693
Wang Z, Zhang D, Hu Z, Cheng J, Zhuo C, Fang X, Xing Y (2015) MicroRNA-26a-modified adipose-derived stem cells incorporated with a porous hydroxyapatite scaffold improve the repair of bone defects. Mol Med Rep 12:3345
Wang XW, He XJ, Lee KC, Huang C, Hu JB, Zhou R, Xiang XY, Feng B, Lu ZQ (2016) MicroRNA-221 sponge therapy attenuates neointimal hyperplasia and improves blood flows in vein grafts. Int J Cardiol 208:79
Wang Y, Malcolm DW, Benoit DSW (2017a) Controlled and sustained delivery of siRNA/NPs from hydrogels expedites bone fracture healing. Biomaterials 139:127
Wang H, Xie Z, Hou T, Li Z, Huang K, Gong J, Zhou W, Tang K, Xu J, Dong S (2017b) MiR-125b regulates the osteogenic differentiation of human mesenchymal stem cells by targeting BMPR1b. Cell Physiol Biochem 41:530
Watt FM, Jensen KB (2009) Epidermal stem cell diversity and quiescence. EMBO Mol Med 1:260
Weilner S, Skalicky S, Salzer B, Keider V, Wagner M, Hildner F, Gabriel C, Dovjak P, Pietschmann P, Grillari-Voglauer R, Grillari J, Hackl M (2015) Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. Bone 79:43
Weilner S, Schraml E, Wieser M, Messner P, Schneider K, Wassermann K, Micutkova L, Fortschegger K, Maier AB, Westendorp R, Resch H, Wolbank S, Redl H, Jansen-Durr P, Pietschmann P, Grillari-Voglauer R, Grillari J (2016) Secreted microvesicular miR-31 inhibits osteogenic differentiation of mesenchymal stem cells. Aging Cell 15:744
Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell 75:855
Wilson KD, Hu S, Venkatasubrahmanyam S, Fu JD, Sun N, Abilez OJ, Baugh JJ, Jia F, Ghosh Z, Li RA, Butte AJ, Wu JC (2010) Dynamic microRNA expression programs during cardiac differentiation of human embryonic stem cells: role for miR-499. Circ Cardiovasc Genet 3:426
Wu Q, Chen D, Zuscik MJ, O'Keefe RJ, Rosier RN (2008) Overexpression of Smurf2 stimulates endochondral ossification through upregulation of beta-catenin. J Bone Miner Res 23:552
Wu K, Song W, Zhao L, Liu M, Yan J, Andersen MO, Kjems J, Gao S, Zhang Y (2013) MicroRNA functionalized microporous titanium oxide surface by lyophilization with enhanced osteogenic activity. ACS Appl Mater Interfaces 5:2733

Xie Q, Wang Z, Zhou H, Yu Z, Huang Y, Sun H, Bi X, Wang Y, Shi W, Gu P, Fan X (2016) The role of miR-135-modified adipose-derived mesenchymal stem cells in bone regeneration. Biomaterials 75:279

Xie Q, Wei W, Ruan J, Ding Y, Zhuang A, Bi X, Sun H, Gu P, Wang Z, Fan X (2017) Effects of miR-146a on the osteogenesis of adipose-derived mesenchymal stem cells and bone regeneration. Sci Rep 7:42840

Xue Y, Ouyang K, Huang J, Zhou Y, Ouyang H, Li H, Wang G, Wu Q, Wei C, Bi Y, Jiang L, Cai Z, Sun H, Zhang K, Zhang Y, Chen J, Fu XD (2013) Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated microRNA circuits. Cell 152:82

Yang F, Green JJ, Dinio T, Keung L, Cho SW, Park H, Langer R, Anderson DG (2009) Gene delivery to human adult and embryonic cell-derived stem cells using biodegradable nanoparticulate polymeric vectors. Gene Ther 16:533

Yau WW, Rujitanaroj PO, Lam L, Chew SY (2012) Directing stem cell fate by controlled RNA interference. Biomaterials 33:2608

Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17:3011

Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, Fuchs E (2006) Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. Nat Genet 38:356

Yi R, Poy MN, Stoffel M, Fuchs E (2008) A skin microRNA promotes differentiation by repressing 'stemness'. Nature 452:225

Yihai C (2003) Angiogenesis inhibitors and their therapeutic potentials. Adv Exp Med Biol 532:109

Yin Q, Gao Y, Zhang Z, Zhang P, Li Y (2011) Bioreducible poly (beta-amino esters)/shRNA complex nanoparticles for efficient RNA delivery. J Control Release 151:35

Yoo AS, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, Lee-Messer C, Dolmetsch RE, Tsien RW, Crabtree GR (2011) MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476:228

Yoshizuka M, Nakasa T, Kawanishi Y, Hachisuka S, Furuta T, Miyaki S, Adachi N, Ochi M (2016) Inhibition of microRNA-222 expression accelerates bone healing with enhancement of osteogenesis, chondrogenesis, and angiogenesis in a rat refractory fracture model. J Orthop Sci: Off J Jpn Orthop Assoc 21:852

Yuan X, Liu H, Huang H, Liu H, Li L, Yang J, Shi W, Liu W, Wu L (2016) The key role of canonical Wnt/β-catenin signaling in cartilage chondrocytes. Curr Drug Targets 17:475

Zeng Y, Cullen BR (2003) Sequence requirements for micro RNA processing and function in human cells. RNA 9:112

Zhang L, Webster TJ (2009) Nanotechnology and nanomaterials: promises for improved tissue regeneration. Nano Today 4:66

Zhang M, Ishii A, Nishiyama N, Matsumoto S, Ishii T, Yamasaki Y, Kataoka K (2009) PEGylated calcium phosphate nanocomposites as smart environment-sensitive carriers for siRNA delivery. Adv Mater 21:3520

Zhang S, Chen L, Jung EJ, Calin GA (2010) Targeting microRNAs with small molecules: from dream to reality. Clin Pharmacol Ther 87:754

Zhang Y, Wang Z, Gemeinhart RA (2013) Progress in microRNA delivery. J Control Release 172:962

Zhang Z, Hou C, Meng F, Zhao X, Zhang Z, Huang G, Chen W, Fu M, Liao W (2015) MiR-455-3p regulates early chondrogenic differentiation via inhibiting Runx2. FEBS Lett 589:3671

Zhang X, Li Y, Chen YE, Chen J, Ma PX (2016a) Cell-free 3D scaffold with two-stage delivery of miRNA-26a to regenerate critical-sized bone defects. Nat Commun 7:10376

Zhang Z, Chen W, Zhang Z, Kang Y, Kang Y, Liao W (2016b) MIR-455-3P and HDACs co-regulate chondrogenesis. Osteoarthr Cartil 24:S224

Zhao C, Sun G, Li S, Shi Y (2009) A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat Struct Mol Biol 16:365

Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D (2011) NF-κB dysregulation in microRNA-146a–deficient mice drives the development of myeloid malignancies. Proc Natl Acad Sci U S A 108:9184

Zhou F, Jia X, Yang Y, Yang Q, Gao C, Hu S, Zhao Y, Fan Y, Yuan X (2016) Nanofiber-mediated microRNA-126 delivery to vascular endothelial cells for blood vessel regeneration. Acta Biomater 43:303

Zuo K, Zhi K, Zhang X, Lu C, Wang S, Li M, He B (2015) A dysregulated microRNA-26a/EphA2 axis impairs endothelial progenitor cell function via the p38 MAPK/VEGF pathway. Cell Physiol Biochem 35:477

Part III

Application Routes and Cell Tracking

Cell Sheets for Tissue Engineering Applications

Ram V. Devireddy

Contents

Abstract

During the past couple of decades, significant progress has been made in using cellularized extracellular scaffolds to regenerate various tissues including bone, cartilage, heart, blood vessel, nerve, liver, and many other tissues. However, most of the conventional scaffolds are complicated in construction and do not allow sufficient cell migration to establish adequate cell-cell adhesion, cell-cell communication, and cell-extracellular matrix (ECM) which are all critically important tissue-level functions. To overcome some of the drawbacks associated with traditional scaffold-based tissue engineering, cell sheet engineering has been developed as an alternative approach and is discussed in this chapter.

R. V. Devireddy (✉)
Department of Mechanical Engineering, Louisiana State University, Baton Rouge, LA, USA
e-mail: rdevir1@lsu.edu

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_33

1 Introduction

Well-established methods in tissue engineering routinely use either the insertion of isolated cell suspensions or biodegradable scaffolds to support the growth and development/differentiation of stem cells into organized three-dimensional tissues (Yang et al. 2005). Typically, this process is facilitated by high-density cell seeding in static and dynamic cultures inside bioreactors to establish and enhance critical cell to extracellular matrix (ECM) and cell-cell interactions (Hubbell et al. 1991; Sittinger et al. 1996; Bauer and Muschler 2000; Griffith and Naughton 2002; Sikavitsas et al. 2002; Griffith 2002; Muschler et al. 2004; Kumar et al. 2007a; Chinnasami et al. 2018). The dispersion of seeded cells within the bio-scaffold plays a crucial role in determining the progression of tissue formation and in establishing a 3D cell culture system (Kumar et al. 2007b). Unfortunately, the lack of vascular channels posits that the cells located within the interior of the scaffold have to rely solely on diffusion for solute-nutrient ingress and waste product egress (Avgoustiniatos and Colton 1997). Thus, most of the currently available methods of scaffold-based cell seeding methods are compromised by the lack of access to nutrients and by the depletion of nutrients by cells located near the outer surface rather than in the interior of the bio-scaffolds (Hutmacher 2000; Sistino 2003; Martin et al. 2004; Janssen et al. 2006; Dvir et al. 2006; Khong et al. 2007).

To overcome some of the drawbacks associated with traditional scaffold-based tissue engineering, cell sheet engineering has been developed as an alternative approach for tissue engineering (Yamato et al. 2001; Yamato 2003; Shimizu et al. 2006; Hayashida et al. 2006; da Silva et al. 2007; Akiyama et al. 2007; Matsuda et al. 2007; Koboyashi and Okano 2010; Tang and Okano 2014; Koboyashi et al. 2017; Kim et al. 2017). Using temperature-responsive poly N-isopropyl acrylamide (PIPAAm)-coated tissue culture polystyrene (TCPS) dishes, cultured cells were successfully harvested as intact sheets by simple temperature changes, thereby avoiding the use of proteolytic enzymes – see Fig. 1. Cell sheet engineering has the advantage of eliminating the use of biodegradable scaffolds. In a recent study, a novel method, using a thermoresponsive hydrogel coated on TCPS dishes, was developed for harvesting living cell sheets (Yamato and Okano 2004; Chen et al. 2006). The hydrogel was prepared by pouring aqueous methylcellulose blended with distinct salts on TCPS dishes at room temperature and subsequently gelled at 37 °C for cell culture (Chen et al. 2006). Methylcellulose (MC) with a viscosity of 4000 cP at 2% solution was used to form gels at physiologically relevant temperatures. However, the procedure described was complex and did not consistently produce stable hydrogels as the MC formulation used was found to be too viscous to be manipulated easily (Chen et al. 2006). Besides, due to the high viscosity of the MC formulation, it is difficult to spread MC hydrogel evenly onto tissue culture dish and therefore resulted in nonuniform coating and a subsequent unstable hydrogel system. This is a major shortcoming

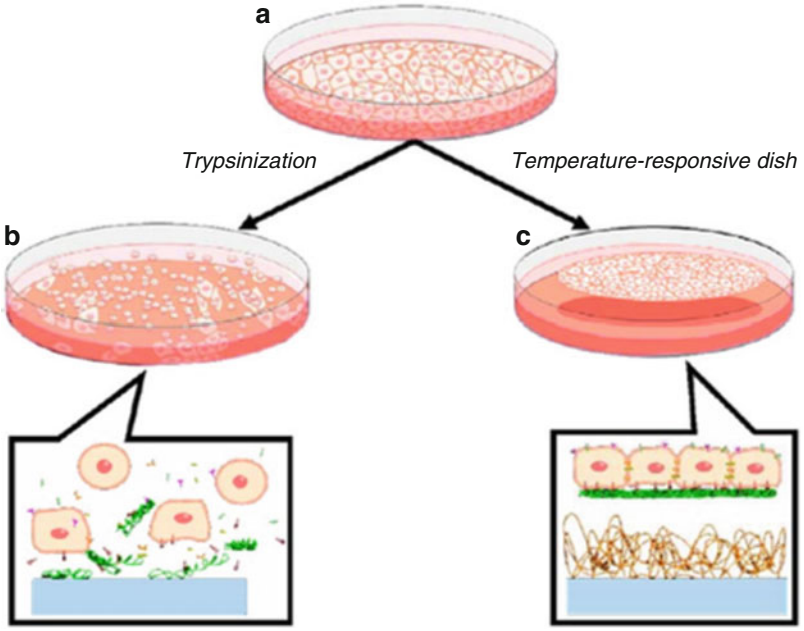


Fig. 1 Schematic illustrations of temperature-responsive cell sheet fabrication vs. traditional trypsinization methods

given that the primary criteria for cell sheet engineering applications are that the MC hydrogel systems generated be consistently stable as the time of culture increases. To address the shortcomings associated with the earlier MC-based cell sheet engineering technology by Chen et al. (2006), Thirumala et al. (2013) developed a modified MC-collagen hydrogel method to create cell sheets, specifically tailored to work with adipose tissue-derived stromal/stem cells (ASCs) and for the creation of multidimensional cell sheets. This process has been modified and enhanced by Forghani et al. (2017) and by Forghani and Devireddy (2018). Forghani et al. (2017) fabricated cell sheets using methylcellulose (MC) gel and with (PNIPAAm) and subsequently characterized ASC sheet formation, cell morphology, viability, proliferation, and differentiation potential over 21 days in parallel studies for direct comparison. In addition, osteogenic differentiation was assessed colorimetrically for alkaline phosphatase (ALP) expression and mineralization, while gene expression for osteocalcin was also assessed by RT-PCR (Forghani et al. 2017). The single or multilayer cell sheets have many applications including tissue reconstruction (Harimoto et al. 2002; Shimizu et al. 2003; Nishida et al. 2004; Akizuki et al. 2005; Ide et al. 2006; Vermette et al. 2007; Obokata et al. 2008; Larouche et al. 2009; Labbe et al. 2011; Fortier et al. 2013; Mota et al. 2015).

2 Adipose Tissue-Derived Adult Stem Cells

Stem cells are essential building blocks of multicellular organisms and provide a valuable tool for the study of cellular and developmental processes. Harnessing the enormous potential of stem cells to differentiate along multi-lineage pathways and produce more specialized cells of the body would provide a tool to study the early human embryonic development and also to develop novel strategies of organ regeneration and transplantation. The stem cell research field has been transformed in the past few years by successes achieved in culturing early human embryonic stem cells and human embryonic germ cells and in manipulating their differentiation in vitro. More recently, excitement has been fueled by evidence that adult stem cells have a higher degree of developmental potential and plasticity, than previously imagined (Pittenger et al. 1999; Gimble and Guilak 2003; Gimble and Nuttall 2004). Though embryonic stem cell potential is significant, the legal and ethical issues associated with embryonic stem cells and their propensity to form malignant teratomas after injection into animal models have refocused attention on adult stem cell models.

Adult stem cells have been isolated from many organs. The central nervous system yields neuronal stem cells that can differentiate and produce not only neuronal cells but also other tissues, including blood and muscle. Stem cells from the liver can be converted, in vitro, into insulin-secreting pancreatic cells. Until recently, bone marrow has been the major source of the adult stem cells. Bone marrow biopsies are invasive and painful procedures and yield volumes of tissue measured in milliliter quantities. Although advances in cytokine technology make it possible to mobilize stem cells from the marrow into the peripheral circulation for easier access, new research has demonstrated that adult stem cells similar to the bone marrow mesenchymal stem cells are plentiful in human fat available via liposuction harvest which, in contrast, routinely delivers liter volumes of tissue. The adipose tissue-derived progenitor cells exhibit multi-lineage potential and express biochemical markers and feature in vitro consistent with bone, cartilage, epithelial, fat, liver, muscle, and nerve cells (Gimble et al. 2007). A study by Hicok et al. (2004) and confirmed by Justesen et al. (2004) showed that these cells derived from human fat are capable of forming osteoid in vivo once implanted subcutaneously into mice. Moreover, human adipose-derived stem cells have been used successfully to repair a damaged region in a skull. In order to harness the potential therapeutic benefits of adipose-derived stem cells (ASCs) for tissue engineering strategies and cell-based therapies, we will require efficient method of delivery and harvesting of these cells in vivo without loss of their multipotentiality.

3 Fabrication of Adult Stem Cell Sheets

Extracts of subcutaneous adipose tissue need to be acquired from several donors through liposuction. In all of the results reported in this chapter, the tissues were obtained from LaCell LLC (New Orleans, La) with informed consent under a clinical

protocol approved by either the Pennington Biomedical Research Center Institutional Review Board (Baton Rouge LA) or Western Institutional Review Board (Puyallup, WA). The ASC isolation procedure has been previously described (Zuk et al. 2001; Gimble and Guilak 2003; Aust et al. 2004; Thirumala et al. 2005a, b; Mitchell et al. 2006; Gimble et al. 2007; Shaik et al. 2017; Chinnasami et al. 2018). Briefly, liposuction aspirates from subcutaneous adipose tissue are obtained from male and female subjects undergoing elective procedures in local plastic surgical offices. Tissues are washed three to four times with phosphate buffered saline and suspended in an equal volume of PBS supplemented with 1% bovine serum and 0.1% collagenase type I pre-warmed to 37 °C. The tissue is then placed in a shaking water bath at 37 °C with continuous agitation for 60 min and centrifuged for 5 min at 300–500× g at room temperature. The supernatant, containing mature adipocytes, is aspirated. The pellet is identified as the stromal vascular fraction (SVF) and suspended/plated immediately in T225 flasks in stromal medium (DMEM/F 12 Ham's, 10% fetal bovine serum (Hyclone, Logan, UT), 100 U penicillin/100 μg streptomycin/0.25 μg Fungizone) at a density of 0.156 ml of tissue digest/sq cm of surface area for expansion and culture. The primary cell culture's initial passage is referred to as "Passage 0" and denoted as P0. The cells are removed from the dish using 5 mL trypsin followed by incubation at 37 °C for 7–10 min and plated at a density of 5000 cells/cm^2 ("Passage 1") in T125 flasks for expansion and allowed to grow to 80% confluency. Passage 2 cells were used for cell viability testing and osteogenesis (Forghani et al. 2017).

Nunc™ Dishes with UpCell™ surface: One method is to plate the ASCs on a thermoresponsive 3.5 cm culture dish (Nunc™ Dishes with UpCell™ Surface, Thermo Scientific). Briefly, 1 mL of pre-warmed stromal medium (37 °C) was added to the culture dish at a cell density of 1×10^6 cells per mL. The culture dish is then to be placed on a hot plate (37 °C) for 30 min for cell attachment. After this 30-min incubation, an additional 1 mL of pre-warmed stromal medium is to be added. The cells are then to be incubated at 37 °C in 5% CO_2 for a further 24 h. The culturing dish needs to be taken out of the incubator after 24 h, and the cell sheet detaches spontaneously after incubation at room temperature for a period of 15–20 min (Fig. 2).

Cell sheets using methylcellulose hydrogel-coated dishes: Fabrication of cell sheets using methylcellulose (MC) hydrogel-coated dishes can also be performed as described by Thirumala et al. (2013) based on prior work by Sarkar (1979), Sarkar and Walker (1995), Ford (1999), Hirose et al. (2000), Tate et al. (2001), Li et al. (2002), Zheng et al. (2004), and Tanga et al. (2007). Briefly, 14% aqueous MC (viscosity 15 cp) solutions are prepared by dissolving commercially available MC powder in heated water with the addition of PBS at room temperature. After being thoroughly shaken and mixed, the solutions are refrigerated overnight until homogeneous MC solutions are obtained. Approximately 500 μL of the 14% MC solution is then poured into the center of each TCPS (3.5 cm) dish placed on ice. A thin transparent layer of the solution needs to be evenly distributed on the TCPS dish. Subsequently, the TCPS dish is to be incubated at 37 °C for 45 min to form an opaque gelled layer. To improve cell attachment, 1 mL of aqueous collagen type I

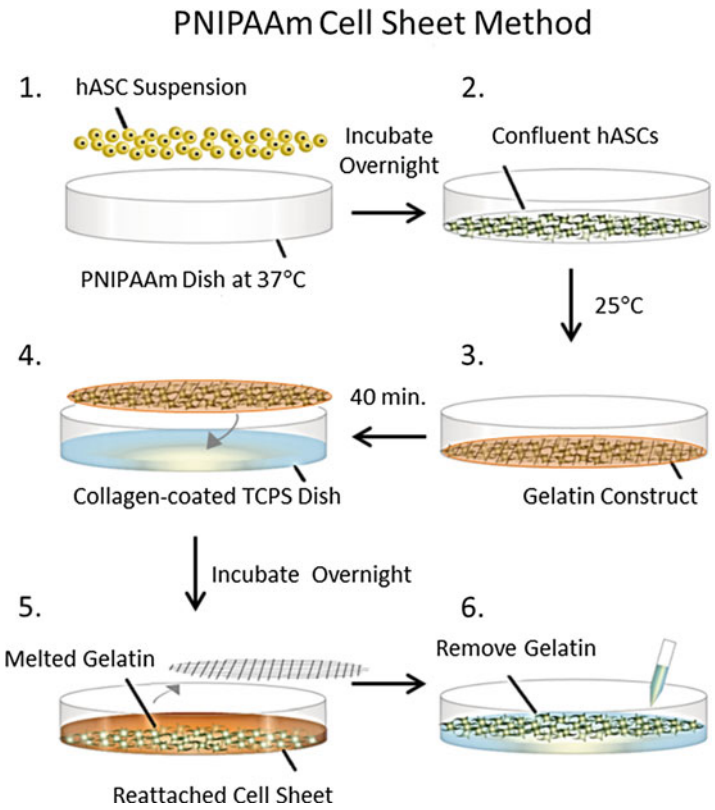


Fig. 2 Schematic illustrations of cell sheet fabrication, detachment, transfer, and reattachment methods using PNIPAAm. ASCS cell sheet formed after overnight incubation using PNIPAAm. (Redrawn with permission from Forghani et al. (2017))

(3 mg/ml, rat tail collagen type I, Sigma-Aldrich) needs to be evenly spread over the gelled MC at 37 °C. Approximately 1.0×10^6 ASCs are then seeded evenly on the MC-coated TCP culture system on a hot plate (37 °C), and a cell sheet will be formed after culturing for 24 h at 37 °C, 5% CO_2. When placed at room temperature (20 °C), the cell sheet detaches gradually from the MC surface (Fig. 3).

4 In Vitro Degradation, Swelling, and Osmotic Stability of MC Gels

Thirumala et al. (2013) assessed the initial weight of the gelled MC hydrogel layer and denoted it as *MC weight*. Subsequently, to investigate the degradation and swelling behavior of the gelled MC hydrogel layer, approximately 2 mL of cell culture media (DMEM-F12 and 10% fetal bovine serum) was added to the TCPS dishes. The weight of the gelled MC hydrogel layer immediately (~10 s) after the

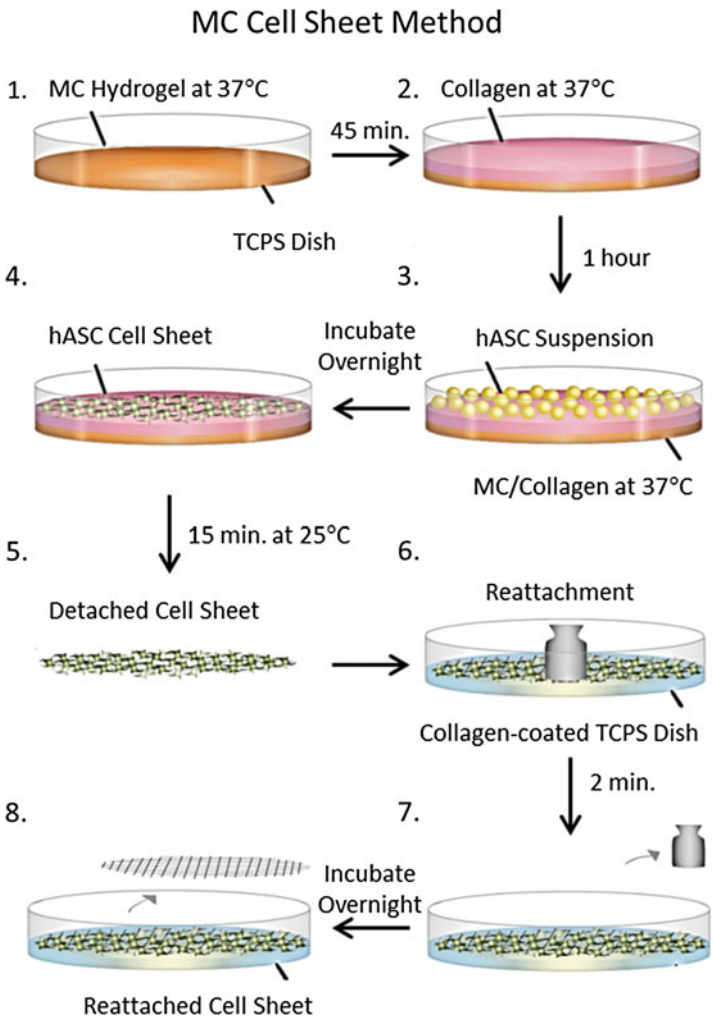


Fig. 3 Schematic illustrations of cell sheet fabrication, detachment, transfer, and reattachment methods using MC. MC hydrogel was fabricated in a TCPS dish followed by coating with collagen. Intact cell sheet was formed after overnight incubation. Cell sheet was transferred to a collagen-coated TCPS dish. A metal mesh disc and weight are placed on top to facilitate reattachment. (Redrawn with permission from Forghani et al. 2017)

addition of cell incubation media was also measured and denoted as "Wet MC weight @ t = 0." The TCPS dishes with the 2 mL of media were then incubated at 37 °C for several days; subsequently, the cell culture media is removed from the TCPS dishes at various time (t) points (1, 4, 7, and 10 days) of incubation, and the weight of the MC hydrogel was recorded and denoted as "Wet MC weight @ t." The MC hydrogel layers were then dried overnight in a convective heater (VWR

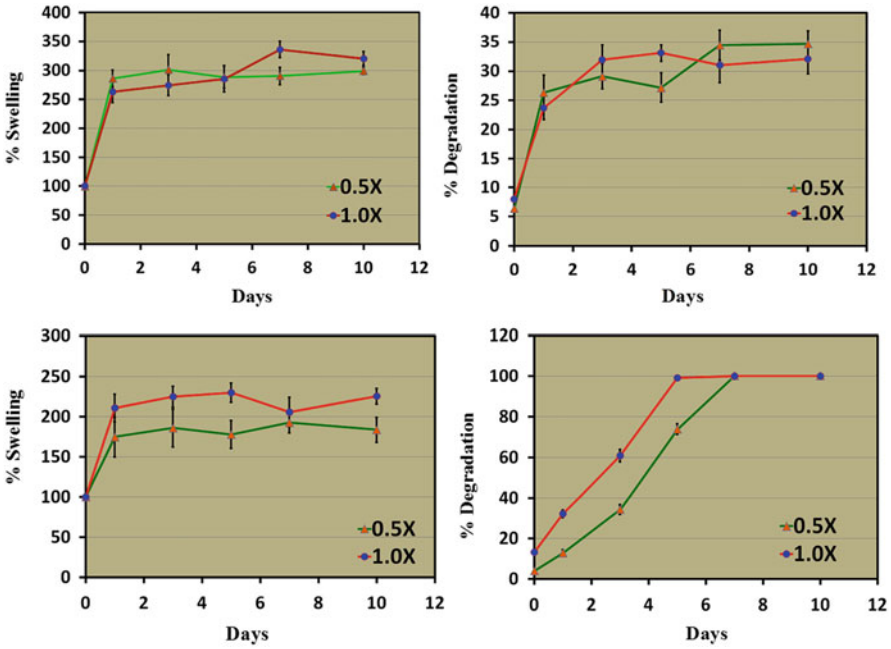


Fig. 4 Percent swelling and percent degradation values of MC (mol. wt = 15,000) system as determined in our laboratory – top 2 figures. Percent swelling and percent degradation values of hydrogel system developed by Chen et al. (*45*) – bottom two figures. Note the different values on the y-axis between the top and bottom figures

Scientific, Bridgeport, NJ, USA, 1602 HAFO series) at 70 °C, and the dried MC weight was recorded and denoted as "Dried MC weight @ t." The percent of MC swelling was quantified, as a function of time (t) as a ratio of "Wet MC weight @ t" to "Wet MC weight @ t = 0," while the percent of degradation was quantified as the ratio of the difference between "Dried MC weight @ t = 0" and "Dried MC weight @ t" to the "Dried MC weight @ t = 0." The results are shown in Fig. 4.

5 Thermal Reversibility of Attachment

Upon confluence, a continuous monolayer ASC sheet was formed on the surface of the MC hydrogel system (Fig. 5). When the confluent cell sheet was removed from the incubator and exposed to room temperature (~30 °C), it controllably detached from the surface of the thermoresponsive hydrogel (without the use of any detachment enzymes, like trypsin). The ASC cell sheet started detaching from its edge at ~1 min after being exposed to room temperature. Detachment of the entire cell sheet was completed within 2–3 min (Fig. 6). Additional control experiments with just a TCPS dish coated with collagen did not result in the formation and spontaneous detachment of an ASC sheet (data not shown). All of the proposed experiments will be conducted using detached ASC sheets (Fig. 7). As these cells were never exposed

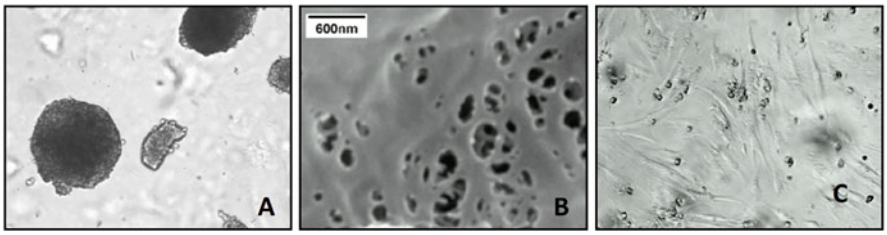


Fig. 5 (**a**) ASCs grown on MC hydrogel system. (**b**) SEM image of the collagen network formed onto the MC-coated TCPS dish. (**c**) ASCs grown on MC hydrogel system coated with collagen

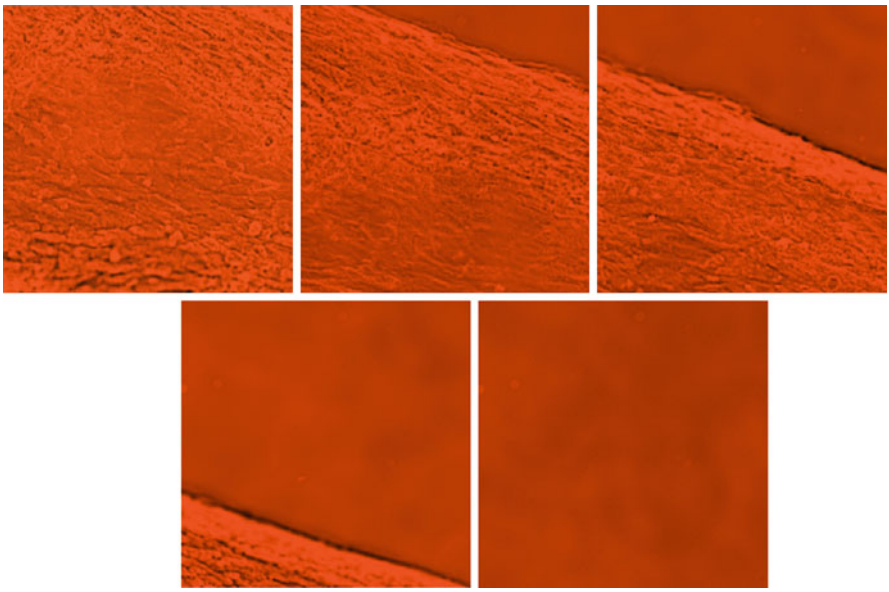


Fig. 6 A series of photomicrographs of the detaching ASC cell sheet with time (20×). The cell sheet detached rapidly (~1 min) after it was transferred to room temperature (~30 °C) from the incubator (~37 °C)

to trypsin and other detachment enzymes, it is to be expected that the binding integrins are not compromised in the cell culture/growth process.

6 Analysis of Cell Sheets Using Histology

Following detachment, ASCs' cell sheets were processed and stained as described previously. Briefly, the cell sheets were rinsed with DPBS, fixed in 10% formalin for 24 h, and embedded in paraffin wax for sectioning. To evaluate the composition of the cell sheets, 10 μm sections were cut and stained with hematoxylin and eosin (H&E) and Masson's trichrome (American MasterTech, Lodi, CA, Item No. KTTRBPT) according

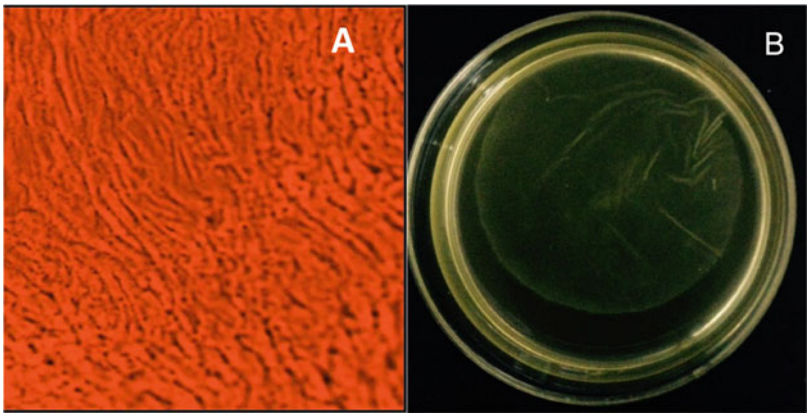


Fig. 7 (**a**) Image of a detached ASC sheet obtained with the MC-collagen hydrogel system exhibiting the characteristics of a monolayer at ~32 °C. (**b**) Floating cell sheet detached from plate

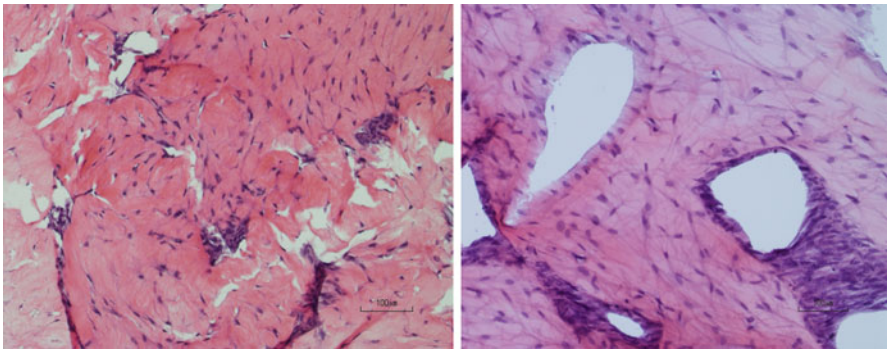


Fig. 8 Histology analysis of cell sheets fabricated with MC (left image) and PNIPAAm (right image) using hematoxylin and eosin (H&E) and trichrome blue staining

to the manufacturer's protocols and imaged under bright-field illumination with an Olympus BX46 microscope at 10× magnification (Fig. 8). Collagen appears pink with H&E and blue with Masson's trichrome stains. Both H&E and Masson's trichrome blue stains confirmed the existence of a considerable amount of collagen in addition to ASCs on MC cell sheets, while PNIPAAm sheets are comprised primarily of ASCs.

7 Cell Viability in Cell Sheets

The cell viability was analyzed over 21 days of culture in stromal medium. PicoGreen® total DNA quantification was used to determine the number of ASCs in cell sheets at day 1, 7, 14, and 21. DNA content analysis indicates that ASCs in

MC cell sheets proliferated until reaching confluence by day 14 and proliferation decreased after day 14, while ASCs in PNIPAAm cell sheets continued to proliferate until day 21 (Forghani et al. 2017).

There was a significant difference * $p < 0.05$ in cell number between day 1 and day 14 in MC sheets, but no significant difference between day 1 and day 21. Conversely, statistical analysis of PNIPAAm cell sheets revealed a significant difference between days 1 and 14 as well as days 1 and 21, with * $p < 0.05$ and ** $p < 0.01$, respectively. ASC viability was also visualized with LIVE/DEAD® staining, representative images of cell sheets at day 1, 7, 14, and 21 with live cells in green and dead cells in red. The LIVE/DEAD® staining indicates that ASCs in both MC and PNIPAAm cell sheets were viable and proliferated until reaching confluence. Cells retained a healthy spindle-shaped morphology in cell sheets (Fig. 9). Images from LIVE/DEAD® staining support the data generated from DNA content analysis that indicate the ASCs in MC cell sheets proliferated until reaching confluence by day 14 and the proliferation stopped at day. ASCs in PNIPAAm cell sheets appear to continue to proliferate until day 21, in agreement with DNA content analysis. Analysis of LIVE/DEAD® staining also shows ASC migration/reorganization over time. It was observed that ASCs in both MC and PNIPAAm cell sheets migrated to achieve a regular distribution of cells throughout the cell sheet and on the culture dish.

8 Confocal Microscopy

MC and PNIPAAm cell sheets were stained to show F-actin distribution in ASCs 1 day after formation. Representative images are shown in Fig. 10. MC cell sheets composed of a collagen layer with ASCs had more depth in a three-dimensional structure compared to the more two-dimensional PNIPAAm cell sheets composed only of ASCs and their ECM. F-actin in MC cell sheets, imaged as a z-stack, appeared to be less organized as cells migrated into the three-dimensional structure of the collagen gel compared to ASCs in PNIPAAm cell sheets which had a highly aligned F-actin structure.

9 Osteogenic Potential of ASCs Embedded in Cell Sheets

Extensive literature exists and has demonstrated that ASCs exhibit differentiation potential and an immunophenotype based on flow cytometric analysis resembling to that of bone marrow-derived mesenchymal stem cells (Halvorsen et al. 2001; Sen et al. 2001; Zuk et al. 2001, 2002; Wickham et al. 2003; Gimble 2003; Thirumala et al. 2010a, b, c; Qureshi et al. 2013; Shaik et al. 2017; Forghani et al. 2017; Chinnasami et al. 2018). Dr. Gimble, in collaboration with Dr. Guilak at the Duke University, developed conditions that promote ASC differentiation along the chondrocyte and osteoblast pathways (Mizuno et al. 2002; Gimble 2003; Awad et al. 2004; Seo et al. 2005). When suspended in calcium alginate and incubated in the

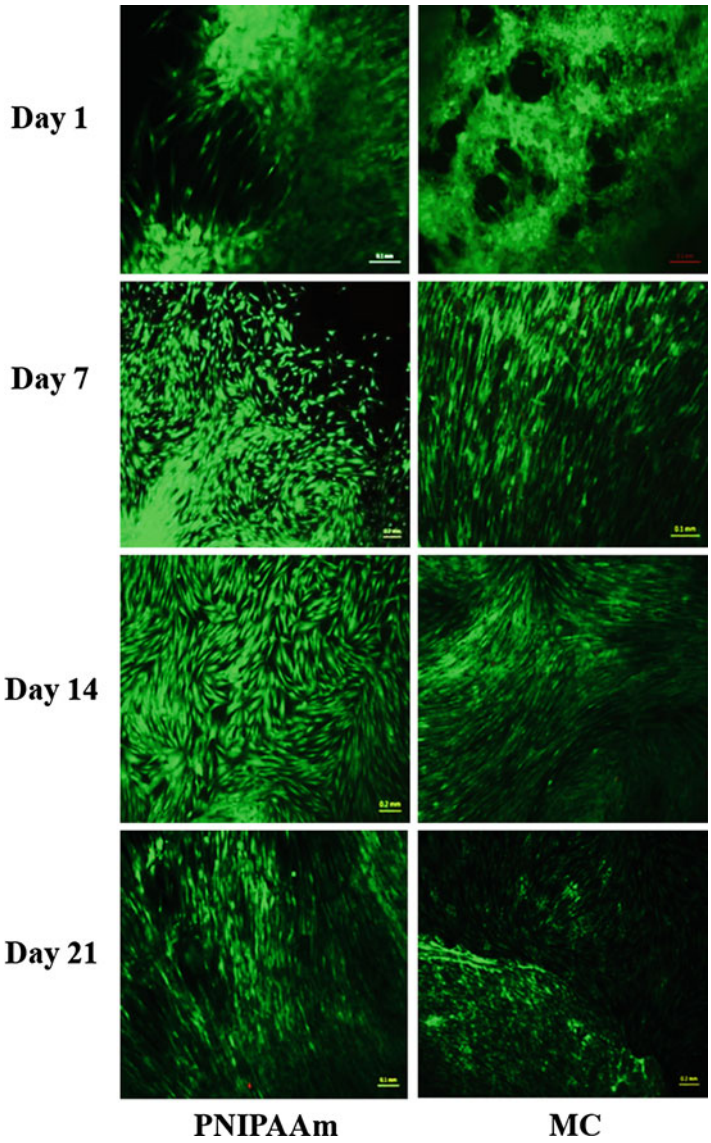


Fig. 9 LIVE/DEAD® staining assessment over 21-day culture period. LIVE/DEAD® assay stains live cells green and dead cells red. Representative overlay images of MC and PNIPAAm cell sheets at day 1, 7, 14, and 21

presence of ascorbate, dexamethasone, and TGF-β, human ASCs exhibit an induction in chondrogenic markers, including collagen types II and VI and proteoglycans. When cultured in the presence of 1,25 dihydroxyvitamin D_3, dexamethasone, ascorbate, and β-glycerophosphate, human ASCs secrete osteocalcin and mineralize

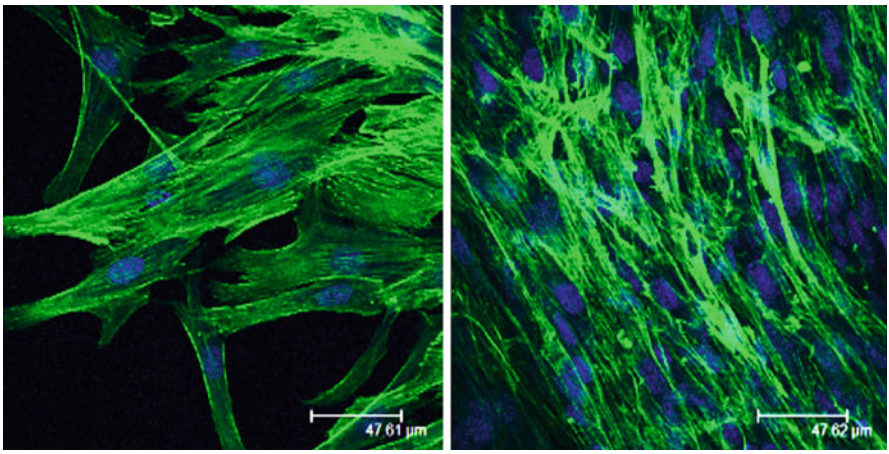


Fig. 10 Confocal images of ASCs in cell sheets 1 day after reattachment. Immunofluorescent staining shows cell nuclei (blue) and F-actin (green) in a representative z-stack of MC cell sheet (left image) and a representative image of PNIPAAm cell sheet (right image)

their extracellular matrix, hallmarks characteristic of osteoblast function. In vivo, human ASCs combined with a hydroxyapatite biomaterial synthesize osteoid matrix when implanted subcutaneously into immunodeficient mice (Justesen et al. 2004).

The ability of ASCs to mineralize extracellular matrix was quantified using Alizarin Red S staining. ASCs from 3 to 5 donors will be plated at 50,000 cells per well in 24-well plates in triplicate and incubated for 24 h in maintenance medium. Cells are then fed either maintenance medium or osteoblast medium composed of DMEM/F-12 Ham's with 10% FBS, 10 mM μ-glycerophosphate, 50 μg/ml sodium ascorbate 2-phosphate, and 100 U penicillin/100 μg streptomycin/0.25 μg Fungizone. Cultures are fed with fresh maintenance medium or osteogenic induction medium every 3–4 days for a period of up to 3 weeks. Cells are fixed in neutral buffered formalin for 1 h and then stained for alkaline phosphatase, using fast blue BB naphthol AS-MX as substrate as previously described (Thirumala et al. 2010a, b, c; Qureshi et al. 2013). Alkaline phosphatase levels are quantitated by measuring trypan blue dye levels in individual wells, using the Bio-Quant Image Analysis program (BioQuant Image Analysis, Nashville, TN). To determine the potential to mineralize extracellular matrix, ASCs are plated in 24-well plates at 20,000 cells per well and incubated for 24 h in maintenance medium. Cells are fed either maintenance medium or osteoblast medium for 21 days. Cells are fixed in 70% ice-cold ethanol and stained with 40 mM Alizarin Red staining on cultures 21 days after the induction of osteogenesis. The percentage area staining positive can then be quantified by scanning the plate and then analyzing the MetaVue software image analysis program. Alternatively, or in combination, we can measure the alkaline phosphatase enzyme activity in cultures at 7 days under osteogenic conditions and normalize the degree of substrate conversion to the number of cells based on DNA content. Both approaches, in

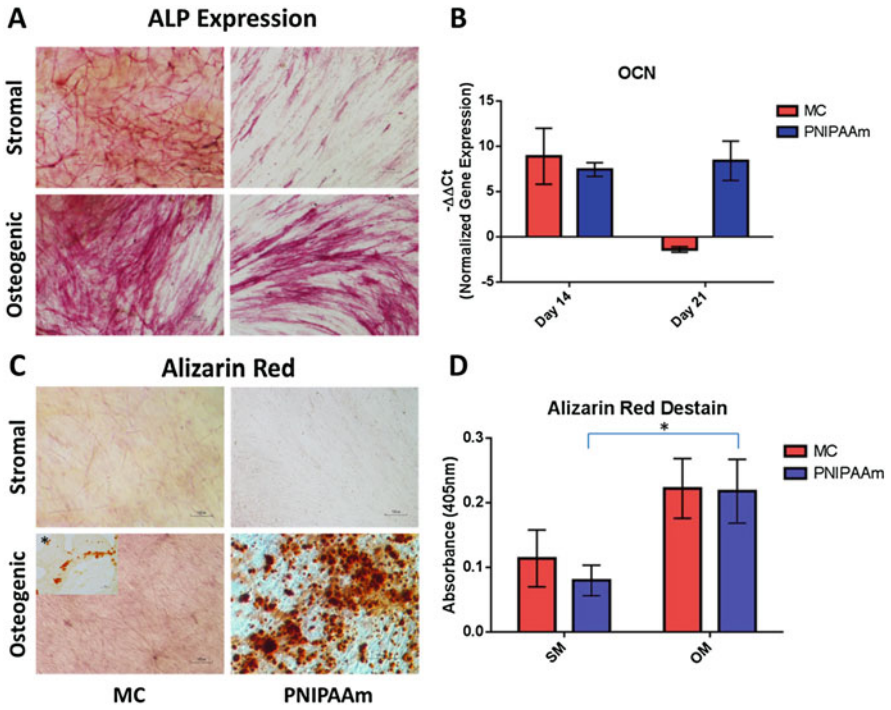


Fig. 11 Markers of osteogenesis of ASCs in MC and PNIPAAm cell sheets. (**a**) ALP expression of ASCs on day 7 cultured in stromal compared to osteogenic conditions. (**b**) Upregulation of OCN in cell sheets at days 14 and 21 cultured in osteogenic conditions compared to stromal conditions. Error bars represent the standard error of the mean. (**c**) Calcium deposition in osteogenic cell sheets at day 21 stained by Alizarin Red. (**d**) Destained cell sheets read at an absorbance of 405 nm to semiquantitatively analyze Alizarin Red. (Reprinted with permission from Forghani et al. 2017)

combination, will provide a measure of osteoblast function in a quantitative manner, as presented recently (Forghani et al. 2017).

To demonstrate the osteogenic potential of ASCs cultured in sheets using MC and PNIPAAm methods, cell sheets were cultured in osteogenic medium for 21 days and compared to ASC cell sheet controls cultured in stromal medium. Osteogenesis results are summarized in Fig. 11. Upregulation of ALP expression was observed in osteogenic cell sheets at day 7. At day 21, cell sheets were stained with Alizarin Red to show calcium deposition. Moderate mineralization was observed in MC cell sheets, compared to the more substantial mineralization of PNIPAAm cell sheets. Cell sheets were destained to allow semiquantitative analysis of Alizarin Red results. In general, an increase in calcium deposition was observed in osteogenic samples relative to those cultured in stromal media for cell sheets prepared by both methods. However, the difference was only statistically significant for PNIPAAm sheets. Due to higher background staining in stromal controls, the increase in Alizarin Red was not statistically significant. For further validation of osteogenesis, OCN expression

was also quantified via RT-PCR at days 14 and 21. There was an increase in OCN expression of MC cell sheets cultured in osteogenic conditions compared to stromal conditions at day 14, with a -ΔΔCt value of 8.895. OCN expression of PNIPAAm cell sheets was upregulated at days 14 and 21 – with an increased -ΔΔCt value from 7.435 at day 14 to 8.395 at day 21.

10 Clinical Problem

Musculoskeletal defects restrict the lives of millions of Americans (Praemer et al. 1999; Kruyt et al. 2004) and require orthopedic surgeries to accelerate or improve bone repair. It is clear that autologous bone does not accommodate every clinical situation and there is a need for bone grafting materials and stem cells in orthopedic surgery (Gupta et al. 2015). Osteoinductive growth factors such as bone morphogenetic proteins are now commercially available (Infuse™ from Sofamor/Medtronics), but they require the presence of osteogenic cells within the spinal fusion site to promote new bone formation. The development of an osteogenic stem cell has the potential to improve the outcome when combined with the alternative spinal fusion materials.

Soft tissue defects represent a second opportunity for the use ASCs in regenerative medicine. The plastic surgery industry had a booming year of more than $14 billion spent on surgical and nonsurgical procedures. This comes to a total of almost 14 million procedures over the year. Of those procedures, almost two million were surgical procedures with liposuction as the top procedure for 2016. With the combination of liposuction and abdominoplasty, which had close to 182,000 procedures, total procedure numbers go into nearly 600,000. This is a total of $83.42 million worth of surgical procedures of the total $8.6 billion spent by Americans for cosmetic surgeries. Many of these surgeries require graft tissue, ideally from the patients themselves to alleviate transplant rejection and immunological rejection response. Additionally, the American Cancer Society reports that over 200,000 women will be diagnosed with new cases of breast cancer each year. A high percentage of these women will undergo partial or complete mastectomy in the course of their treatment. Plastic and reconstructive surgeons are seeking novel methods to repair the resulting soft tissue defects in these patients. We postulate that adipogenic ASCs, in combination with cell sheet-engineered scaffolds, may prove to be a cell-based therapeutic tool for oncological surgeons. All of these procedures have the potential of needing fresh adipose tissue on hand for further procedures or to consider preservation of tissue with the idea of harvesting adipose-derived stem cells. As an aside, recent studies suggest that ASC cryopreservation was associated with a reduced adipogenic capacity in vitro and osteogenic differentiation in vitro and in vivo (Hebert et al. 2009; James et al. 2010), thus necessitating further studies in optimizing the freeze-storage process for the tissue constructs. Given the two-dimensional nature of the cell sheet-engineered materials, the complexity of freezing process optimization is reduced when compared to the

complexity associated with three-dimensional large-scale traditional bio-scaffold-based tissue constructs.

11 Conclusion

Several techniques are currently available for cell sheet fabrication (Yamato et al. 2001; Akiyama et al. 2007; Koboyashi and Okano 2010; Tang and Okano 2014; Koboyashi et al. 2017; Kim et al. 2017). The two techniques demonstrated in this chapter (Thirumala et al. 2013; Forghani et al. 2017; Forghani and Devireddy 2018) allow users to transfer and manipulate the cell sheets in vitro while maintaining sheet morphology, ECM structure, and cell-cell interactions largely intact. Cell sheets fabricated by using MC or PNIPAAm methods maintained cell viability for up to 21 days. It is clear that ASCs' cell sheets made by either MC or PNIPAAm have potential as a research tool to examine complex cell-cell interactions and for potential clinical use in tissue engineering applications. Cell sheets made by PNIPAAm method demonstrated greater ossification compared with the MC method, indicating the PNIPAAm fabrication approach may be better suited for application in bone tissue engineering research. Future studies will explore the potential of cell sheets fabricated by both methods for different applications in tissue engineering (Costa et al. 2017; Baksh et al. 2017; Sudo et al. 2018).

Acknowledgment The authors thank Dr. Elizabeth Clubb and Dr. James Wade at the Pennington Biomedical Research Center (PBRC) for supplying the liposuction aspirates and their many patients for consenting to participate in this protocol. Acknowledgements are also due to Dr. Gimble, a longtime colleague and friend, without his help this work would never have been initiated. And finally, acknowledgements are also due to Dr. S. Thirumala and A. Forghani for performing the seminal experiments reported in this chapter. This work was supported in part by funding from the Louisiana Board of Regents, the Department of Mechanical Engineering at the Louisiana State University (LSU) as well as the NSF EPSCoR LA-SiGMA project under award #EPS-1003897.

References

Akiyama Y, Kushida A, Yamato M, Kikuchi A, Okano T (2007) Surface characterization of poly (n-isopropylacrylamide) grafted tissue culture polystyrene by electron beam irradiation, using atomic force microscopy, and x-ray photoelectron spectroscopy. J Nanosci Nanotechnol 7:796–802

Akizuki T, Oda S, Komaki M, Tsuchioka H, Kawakatsu N, Kikuchi A, Yamato M, Okano T, Ishikawa I (2005) Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. J Periodontal Res 40:245–251

Aust L, Devlin BH, Foster SJ, Halvorsen YDC, Hicok K, Kloster AL, du Laney TV, Sen A, Willingmyre D, Gimble JM (2004) Recovery of human adipose derived adult stem cells from liposuction aspirates. Cytotherapy 6:1–8

Avgoustiniatos ES, Colton CK (1997) Effect of external oxygen mass transfer resistances on viability of immunoisolated tissue. Ann N Y Acad Sci 831:145–167

Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 25:3211–3222

Baksh N, Gallant ND, Toomey RG (2017) Cell sheet engineering for integrating functional tissue in vivo: successes and challenges. Mater Res Soc Bull 42:350–355

Bauer TW, Muschler GF (2000) Bone graft materials: an overview of the basic science. Clin Orthop Relat Res 371:10–27

Chen CH, Tsai CC, Chen W, Mi FL, Liang HF, Chen SC, Sung HW (2006) Novel living cell sheet harvest system composed of thermoreversible methylcellulose hydrogels. Biomacromolecules 7:736–743

Chinnasami H, Gimble JM Devireddy RV (2018) Structure-property relation of porous plla scaffolds fabricated using organic solvent mixtures and controlled cooling rates and its bio-compatibility with human adipose stem cells. J Bioact Biocompatible Polym Biomed Appl 33:397–415 (in press)

Costa M, Cerqueira MT, Santos TC, Sampaio-Marques B, Ludovico P, Marques AP, Pirraco RP, Ruis RL (2017) Cell sheet engineering using the stromal vascular fraction of adipose tissue as vascularization strategy. Acta Biomater 55:131–143

Dvir T, Benishti N, Shachar M, Cohen S (2006) A novel perfusion bioreactor providing a homogenous milieu for tissue regeneration. Tissue Eng 12:2843–2852

Ford JL (1999) Thermal analysis of hydroxypropylmethylcellulose and methylcellulose: powders, gels and matrix tablets. Int J Pharm 179:209–228

Forghani A, Devireddy RV (2018) Methylcellulose based thermally reversible hydrogels. Methods Mol Biol 1773:41–51

Forghani A, Kreigh L, Hogan K, Chen C, Brewer G, Devireddy RV, Hayes D (2017) Fabrication and characterization of cell sheets using methylcellulose and PNIPAAm thermoresponsive polymers: a comparative study. J Biomed Mater Res Part A 105:1346–1354

Fortier GM, Gauvin R, Proulx M, Vallee M, Fradette J (2013) Dynamic culture induces a cell-type dependent response impacting on the thickness of engineered constructive tissue. J Tissue Eng Regen Med 7:292–301

Gimble JM (2003) Adipose tissue-derived therapeutics. Expert Opin Biol 3:705–713

Gimble J, Guilak F (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy 5:362–369

Gimble JM, Nuttall ME (2004) Bone and fat: old questions, new insights. Endocrine 23:183–188

Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100:1249–1260

Griffith LG (2002) Emerging design principles in biomaterials and scaffolds for tissue engineering. Ann N Y Acad Sci 961:83–95

Griffith LG, Naughton G (2002) Tissue engineering: current challenges and expanding opportunities. Science 295:1009–1014

Gupta A, Kukkar N, Sharif K, Main BJ, Albers CE, El-Amin SF (2015) Bone graft substitutes for spine fusion: a brief review. World J Orthop 6(6):449–456

Halvorsen YDC, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM (2001) Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 7:729–741

Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, Okano T (2002) Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. J Biomed Mater Res 62:464–470

Hayashida Y, Nishida K, Yamato M, Yang J, Sugiyama H, Watanabe K, Hori Y, Maeda N, Kukuchi A, Okano T, Tano Y (2006) Transplantation of tissue-engineered epithelial cell sheets after excimer laser photoablation reduces postoperative corneal haze. Invest Ophthalmol Vis Sci 47:552–557

Hebert TL, Wu X, Yu G, Goh BC, Halvorsen YD, Wang Z, Moro C, Gimble JM (2009) Culture effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on cryopreserved human adipose-derived stromal/stem cell proliferation and adipogenesis. J Tissue Eng Regen Med 3:553–561

Hicok K, du Laney T, Sheng Zhou Y, Halvorsen YDC, Hitt CD, Cooper LF, Gimble JM (2004) Human adipose derived adult stem cells produce osteoid in vivo. Tissue Eng 10:371–380

Hirose M, Kwon OH, Yamato M, Kikuchi A, Okano T (2000) Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. Biomacromolecules 1:377–381

Hubbell JA, Massia SP, Desai NP, Drumheller PD (1991) Endothelial cell-selective materials for tissue engineering in the vascular graft via a new receptor. Biotechnology 9:568–572

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

Ide T, Nishida K, Yamato M, Sumide T, Utsumi M, Nozaki T, Kikuchi A, Okano T, Tano Y (2006) Structural characterization of bioengineered human corneal endothelial cell sheets fabricated on temperature-responsive culture dishes. Biomaterials 27:607–614

James AW, Levi B, Nelson ER, Peng M, Commons GW, Lee M, Wu B, Longaker MT (2010) Deleterious effects of freezing on osteogenic differentiation of human adipose-derived stromal cells in vitro and in vivo. Stem Cells Dev 20:427439

Janssen FW, Oostra J, Oorschot A, van Blitteswijk CA (2006) A perfusion bioreactor system capable of producing clinically relevant volumes of tissue-engineered bone: in vivo bone formation showing proof of concept. Biomaterials 27:315–323

Justesen J, Pedersen SB, Stenderup K, Kassem M (2004) Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. Tissue Eng 10:381–391

Khong YM, Zhang J, Zhou S, Cheung C, Doberstein K, Samper V, Yu H (2007) Novel intra-tissue perfusion system for culturing thick liver tissue. Tissue Eng 13:2345–2356

Kim J-H, Ko S-K, Lee JH, Kim D-H, Yun J-H (2017) Evaluation of the periodontal regenerative properties of patterned human periodontal ligament stem cell sheets. J Periodontal Implant Sci 47:402–415

Koboyashi J, Okano T (2010) Fabrication of a thermoresponsive cell culture dish: a key technology for cell sheet engineering. Sci Technol Adv Mater 11:014111

Koboyashi J, Yamato M, Okano T (2017) On-off affinity binding modulation on thermoresponsive polymer-grafted surfaces for captured release of proteins and cells. J Biomater Sci Polym 28:939–957

Kruyt MC, van Gaalen SM, Oner FC, Verbout AJ, de Bruijn JD, Dhert WJ (2004) Bone tissue engineering and spinal fusion: the potential of hybrid constructs by combining osteoprogenitor cells and scaffolds. Biomaterials 25:1463–1473

Kumar PRA, Varma HK, Kumary TV (2007a) Cell patch seeding and functional analysis of cellularized scaffolds for tissue engineering. Biomed Mater 2:48–54

Kumar PRA, Sreenivasan K, Kumary TV (2007b) Alternate method for grafting thermoresponsive polymer for transferring in vitro cell sheet structures. J Appl Polym Sci 105:2245–2251

Labbe B, Marceau-Fortier G, Fradette J (2011) Cell sheet technology for tissue engineering: the self-assembly approach using adipose-derived stromal cells. Methods Mol Biol 702:429–441

Larouche D, Paquet C, Fradette J, Carrier P, Auger FA, Germain L (2009) Regeneration of skin and cornea by tissue engineering. Methods Mol Biol 482:233–256

Li L, Shan H, Yue CY, Lam YC, Tam KC, Hu X (2002) Thermally induced association and dissociation of methylcellulose in aqueous solutions. Langmuir 18:7291–7298

Martin I, Wendt D, Heberer M (2004) The role of bioreactors in tissue engineering. Trends Biotechnol 22:80–86

Matsuda N, Shimizu T, Yamato M, Okano T (2007) Tissue engineering based on cell sheet technology. Adv Mater 19:3089–3099

Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24:376–385

Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH (2002) Myogenic differentiation of human processed lipoaspirate cells. Plast Reconstr Surg 109:199–209

Mota C, Puppi D, Chiellini F, Chiellini E (2015) Additive manufacturing techniques for the production of tissue engineering constructs. J Tissue Eng Regen Med 9:174–190

Muschler GF, Nakamoto C, Griffith LG (2004) Engineering principles of clinical cell-based tissue engineering. J Bone Jt Surg 86:1541–1558

Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T (2004) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo in a temperature-responsive cell culture surface. Transplantation 77:379–385

Obokata H, Yamato M, Yang J, Nishida K, Tsuneda S, Okano T (2008) Subcutaneous transplantation of autologous oral mucosal epithelial cell sheets fabricated on temperature-responsive culture dishes. J Biomed Mater Res A 86:1088–1096

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

Praemer A, Furner S, Rice DP, Kelsey JL (1999) Musculoskeletal conditions in the United States. American Academy of Orthopedic Surgeons, Rosemont

Qureshi AT, Monroe WT, Dasa V, Gimble JM, Hayes DJ (2013) miR-148b–nanoparticle conjugates for light mediated osteogenesis of human adipose stromal/stem cells. Biomaterials 34:7799–7810

Sarkar N (1979) Thermal gelation properties of methyl and hydroxypropyl methylcellulose. J Appl Polym Sci 24:1073–1087

Sarkar N, Walker LC (1995) Hydration-dehydration properties of methylcellulose and hydroxypropylmethylcellulose. Carbohydr Polym 27:177–185

Sen A, Lea-Currie YR, Sujkowska D, Franklin DM, Wilkison WO, Halvorsen YD, Gimble JM (2001) Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. J Cell Biochem 81:312–319

Seo MJ, Suh SY, Bae YC, Jung JS (2005) Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun 328:258–264

Shaik S, Hayes D, Gimble JM, Devireddy RV (2017) Inducing heat shock proteins enhances the stemness of frozen-thawed adipose tissue derived stem cells. Stem Cells Dev 26:608–616

Shimizu T, Yamato M, Kikuchi A, Okano T (2003) Cell sheet engineering for myocardial tissue reconstruction. Biomaterials 24:2309–2316

Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, Kobayashi E, Okano T (2006) Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. FASEB J 20:708–710

Sikavitsas VI, Bancroft GN, Mikos AG (2002) Formation of three-dimensional cell/polymer constructs for bone tissue engineering in a spinner flask and a rotating wall vessel bioreactor. J Biomed Mater Res 62:136–148

da Silva RMP, Mano JF, Reis RL (2007) Smart thermoresponsive coatings and surfaces for tissue engineering: switching cell-material boundaries. Trends Biotechnol 25:577–583

Sistino JJ (2003) Bioreactors for tissue engineering—a new role for perfusionists? J Extra Corporeal Technol 35:200–202

Sittinger M, Bujia J, Rotter N, Reitzel D, Minuth WW, Burmester GR (1996) Tissue engineering and autologous transplant formation: practical approaches with resorbable biomaterials and new cell culture techniques. Biomaterials 17:237–242

Sudo Y, Kawai R, Sakai H, Kikcuhi R, Nabae Y, Hayakawa T, Kakimoto M (2018) Star-shaped thermoresponsive polymers with various functional groups for cell sheet engineering. Langmuir 34:653–662

Tang Z, Okano T (2014) Recent development of temperature-responsive surfaces and their application for cell sheet engineering. Regen Biomater 1:91–102

Tanga Z, Kikuchi A, Akiyamaa Y, Okano T (2007) Novel cell sheet carriers using polyion complex gel modified membranes for tissue engineering technology for cell sheet manipulation and transplantation. React Funct Polym 67:1388–1397

Tate MC, Shear DA, Hoffman SW, Stein DG, LaPlaca MC (2001) Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury. Biomaterials 22:1113–1123

Thirumala S, Gimble JM, Devireddy RV (2005a) Transport phenomena during freezing of adipose tissue derived adult stem cells. Biotechnol Bioeng 92:372–383

Thirumala S, Zvonic S, Floyd E, Gimble JM, Devireddy RV (2005b) The effect of various freezing parameters on the immediate post-thaw membrane integrity of adipose tissue derived adult stem cells. Biotechnol Prog 21:1511–1524

Thirumala S, Gimble JM, Devireddy RV (2010a) Cryopreservation of stromal vascular fraction of adipose tissue in a serum-free freezing medium. J Tissue Eng Regen Med 4:224–232

Thirumala S, Wu X, Gimble JM, Devireddy RV (2010b) Evaluation of polyvinylpyrrolidone as a cryoprotectant for adipose tissue-derived adult stem cells. Tissue Eng Part C Methods 16:783–792

Thirumala S, Gimble JM, Devireddy RV (2010c) Evaluation of methylcellulose and dimethyl sulfoxide as the cryoprotectants in a serum-free freezing media for cryopreservation of adipose-derived adult stem cells. Stem Cells Dev 19:513–522

Thirumala S, Gimble JM, Devireddy RV (2013) Methylcellulose based thermally reversible hydrogel system for tissue engineering applications. Cells 2:60–475

Vermette M, Trottier V, Menard V, Saint-Pierre L, Roy A, Fradette J (2007) Production of a new tissue-engineered adipose substitute from human adipose-derived stromal cells. Biomaterials 28:2850–2860

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

Yamato M (2003) Cell sheet engineering: from temperature-responsive culture surfaces to clinics. In: European cells and materials: biosurface V – functional polymeric surfaces in biotechnology. ETH, Zürich, pp 26–27

Yamato M, Okano T (2004) Cell sheet engineering. Mater Today 7:42

Yamato M, Utsumi M, Kushida A, Konno C, Kikuchi A, Okano T (2001) Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng 7:473–480

Yang J, Yamato M, Kohno C, Nishimoto A, Sekine H, Fukai F, Okano T (2005) Cell sheet engineering: recreating tissues without biodegradable scaffolds. Biomaterials 26:6415–6422

Zheng P, Li L, Hu X, Zhao X (2004) Sol-gel transition of methylcellulose in phosphate buffer saline solutions. J Polym Sci B Polym Phys 42:1849–1860

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benjaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, John K, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue as a source of multipotent stem cells. Mol Biol Cell 13:4279–4295

Seeing Is Believing: Noninvasive Microscopic Imaging Modalities for Tissue Engineering and Regenerative Medicine

Nuno Neto, Ruslan I. Dmitriev, and Michael G. Monaghan

Contents

N. Neto
Department of Mechanical and Manufacturing Engineering and Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland
e-mail: neton@tcd.ie

R. I. Dmitriev
Metabolic Imaging Group, School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland
e-mail: R.Dmitriev@ucc.ie

M. G. Monaghan (✉)
Department of Mechanical and Manufacturing Engineering and Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland

Advanced Materials for BioEngineering Research (AMBER) Centre, Trinity College Dublin and Royal College of Surgeons in Ireland, Dublin 2, Ireland
e-mail: monaghmi@tcd.ie

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_40

Abstract

Microscopy has made a significant impact on science and is today an integral part of a researcher toolbox and techniques to test scientific hypothesis. However, only recently has the possibility to observe important biological interactions and mechanisms deep inside a tissue, with the ability to focus on a single cell, emerged. New technological discoveries have resulted in broadening the focus of this standard method and promoted novel modalities to be used in several biological contexts and to validate scientific hypothesis. Nowadays, noninvasive methods have shown interesting and groundbreaking applications in tissue engineering, tumor biology, and immunology. Multiphoton microscopy (MPM), second harmonic generation (SHG), fluorescence lifetime imaging microscopy (FLIM), Förster resonance energy transfer (FRET), and intravital microscopy (IVM) have harnessed both endogenous and exogenous fluorophores to uncover new molecular and cellular biology pathways while enabling single cell tracking. By means of these techniques, it is now possible to unveil extracellular matrix density and alignment, perform metabolic analysis, observe complex macromolecular interactions, and validate cell division, death, communication, and fate. Within this chapter, we highlight and showcase the versatility and applicability of different microscopic approaches and their qualitative and quantitative insight into cellular and molecular biology, tissue engineering, immunology, metabolism, and tumor biology.

1 Introduction

Since its development in the sixteenth century by Hans and Zacharias Janssen, microscopy rapidly found applications in imaging, and during the seventeenth century, works by Robert Hooke and Anton van Leeuwenhoek reported the first high-magnification visualization of cells (Leeuwenhoek 1682; Hooke 1665). Nowadays, microscopic imaging remains a steadfast tool in the life sciences with microscopy techniques routinely applied in the biosciences and tissue engineering research fields. Transmission light, confocal, and fluorescence microscopy are the most commonly used microscopic techniques for cell identification, analysis, and evaluation of cell response to chemical and mechanical stimuli. However, transmission light microscopy gives generic detail of cell responses and is predominantly

employed to evaluate cell division and proliferation (Zaritsky et al. 2011). Confocal and fluorescence microscopy often require cell or tissue staining using external fluorophores, either alone or bound to antibodies, rendering it an invasive technique as stained cells are often not compatible for further assays (Gurcan et al. 2009). The use of invasive techniques can require pretreatments or fixation regimes which affect cell and tissue physiology and protein conformation, rendering it a destructive endpoint technique (Mariani et al. 2009). Such necessities have generated an increasing demand for noninvasive methods of fluorescence microscopy in order to diagnose diseases and optimize tissue engineering approaches (Quinn et al. 2012). The use of noninvasive fluorescence-based microscopic techniques can allow real-time analysis of living cells without the requirement of cell fixation, reducing sample manipulation and interference while decreasing costs. In this chapter, we discuss emerging noninvasive fluorescent-based imaging technologies in the evaluation of cell and tissue behavior. These applications enable high-resolution in vitro, in situ, and in vivo imaging of ECM structures and cells in intact and engineered tissues. Uncovering the full potential of noninvasive imaging can provide more accurate results and reduce sample manipulation while avoiding the pitfalls of complex and invasive staining protocols. In particular, we focus on the advanced modality techniques of multiphoton microscopy (MPM) and its applications in second harmonic generation (SHG), fluorescence lifetime imaging microscopy (FLIM), Förster resonance energy transfer (FRET), and phosphorescence lifetime imaging microscopy (PLIM).

2 Principle of Fluorescence

The interaction of light with matter is underpinned by quantum theory which is the basis of most microscopy techniques. Due to the higher mass of protons and their strong electromagnetic interaction in the nucleus of an atom, light interacts preferably with electrons, characterized by their lower mass, existing at the periphery of atoms. The electron of an atom or molecule can absorb the energy of an incident photon of light, promoting a transition to a higher energy state – this energy transfer process is called absorption. The energy associated with a photon is described by the Einstein-Planck equation (1), with h being Planck's constant, c the speed of light, and λ the photon wavelength. The energy gap that the electron must overcome in order to jump to the increasing energy state depends on the position of the electron, the atom, and the molecular structure of a compound. This is the basis of all fluorescence microscopy, whereby excitation light of a defined wavelength generates fluorescence emission from an excited fluorophore at an emission wavelength.

$$E = \frac{hc}{\lambda} \tag{1}$$

Before the 1930s, a principle was held that this quantic jump could only occur if the energy of the photon is equal or above the energy gap of the electron transition (Lakowicz 1999). In 1931 this dogma was revised. Maria Goeppert-Mayer described

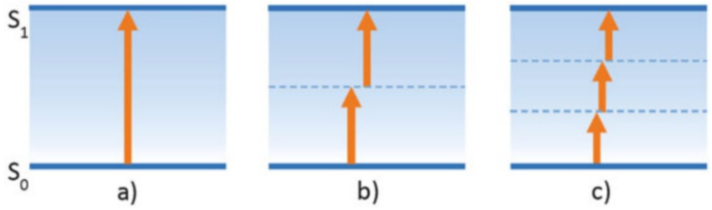


Fig. 1 Energy transitions due to light absorption. This transition occurs from ground state (S_0) to a higher energy state (S_1) and can be promoted by (**a**) one-photon, (**b**) two-photon, or (**c**) three-photon excitation

an alternative process in her doctoral dissertation which hypothesized that in specific conditions, such an excitation process could be achieved in two steps (Göppert-Mayer 1931). Specifically, two photons whose combined energy would be equal or higher to the energy gap could be enough to achieve an electronic transition. According to quantum mechanics, a single photon excites the molecule to a virtual intermediate state that by recombination with a second photon brings the molecule to the final excited state and higher energy level. Only in 1961 was this process first confirmed by Wolfgang Kaiser using a CaF_2:Eu^{2+} crystal laser (Kaiser and Garrett 1961). This quantum process is the basis of MPM (Fig. 1).

3 Multiphoton Microscopy (MPM)

MPM can be modelled further in a time-dependent Schrödinger equation in which the Hamiltonian contains an electric dipole moment:$\vec{E}_\gamma \cdot \vec{r}$, where $\vec{E}_\gamma$ is the electric field vector of the photons and $\vec{r}$ is the position operator. ε_γ is the photonic energy associated with the electric field vector, and ε_m is the energy difference between the state m and the ground state. The first-order solution corresponds to the one-photon excitation, and multiphoton transitions are represented by higher-order solutions. Here, the transition probability between the molecular initial state$|i\rangle$, the virtual intermediate state $|m\rangle$, and the final state $|f\rangle$ is given by:

$$P \sim \left| \sum_m \frac{\langle f|\vec{E}_\gamma \cdot \vec{r}\,|m\rangle\langle m|\vec{E}_\gamma \cdot \vec{r}\,|i\rangle}{\varepsilon_\gamma - \varepsilon_m} \right|^2 \tag{2}$$

After excitation of a molecule, by a single or multiphoton approach to S_1 energy level, the molecule undergoes internal conversion and reaches the lowest vibrational level of the S_1 state. From here the energy can be dispersed in a radiative or non-radiative way followed by a return to its ground state. A radiative decay is associated with light emission phenomena, while non-radiative decay is related to the losing of energy by particle vibration and by heat.

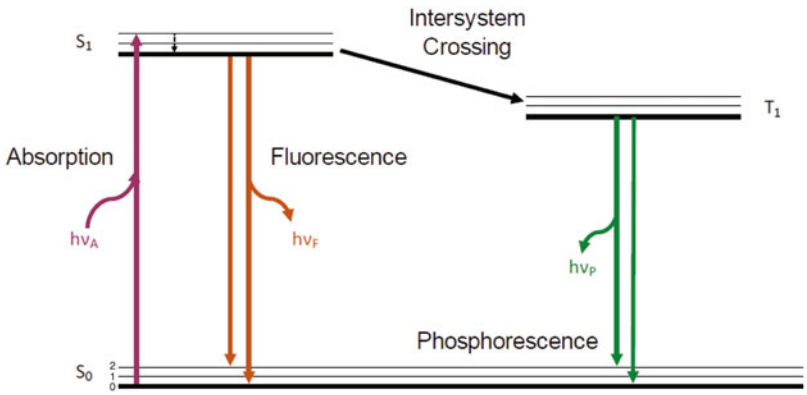


Fig. 2 Simplified Jablonski diagram. Higher vibrational levels are represented by thin lines. $h\nu_{A/F/P}$ is the energy associated to each photon described by Einstein-Planck's equation (1). h is related to the Planck's constant and $\nu_{A/F/P}$ with the wave frequency of each transition process

Radiative decay can be decomposed further into fluorescence or phosphorescence processes. In detail, fluorescence is characterized by short decay time durations (picoseconds to nanoseconds) and occurs from S_1 to S_0 energy level (Fig. 2). Phosphorescence has a longer decay time (micro- to milliseconds) due to intersystem crossing that the molecule must undergo to reach the energy state T_1 and then convert back to S_0.

The radiative decays of an electron are dependent on the molecular structure and its local environment such as solvent, pH, and temperature. The rate of these decays is measured by quantum yields, whereby a high fluorescence quantum yield results in a higher percentage of energy being released by light, subsequently rendering a brighter emission and higher number of photons being radiated. The absorption and decay pathways that an electron or molecule can undergo are graphically represented in a Jablonski diagram (Fig. 2).

In MPM the absorption step in the Jablonski diagram (Fig. 2) is achieved using light amplification by stimulated emission of radiation (LASER). Wavelengths on the ultraviolet (UV), visible, and near infrared (NIR) range are commonly used as excitation sources in microscopy. When going deeper inside of a sample, light scattering dominates the attenuation of light propagation. However, with the use of nonlinear optical processes such as two-photon excitation, the effects of emission scattering are mitigated. In addition, longer wavelengths can be used which increase light penetration and reduce photobleaching due to the confinement of the excitation volume in a tissue due to the quantic nature of the multiphoton recombination. To accomplish this, intense ultrashort pulse lasers in the femtosecond range (~80–250 fs) are used (Zipfel et al. 2003b; Wilt et al. 2009).

Therefore, wavelengths applied in MPM have lower energies, and less scattering of photons occurs when imaging deep inside biological tissues, while less damage to the samples is produced in comparison with conventional confocal/fluorescence microscopy with higher photostability and higher laser penetration potential.

4 Second Harmonic Generation (SHG)

4.1 Principle of SHG

Falling under the domain of MPM, SHG is a nonlinear optical spectroscopic (NLO) method that has been applied to biological microscopy. It enables direct imaging of anisotropic structures containing large hyperpolarization features (Zipfel et al. 2003a). SHG is a quantic phenomenon in which two photons are converted into a single photon at twice the frequency of the individual exciting photons (Stoller et al. 2003). Therefore, SHG can also be defined as a frequency-doubling procedure (Campagnola 2011). SHG possesses common features with multiphoton microscopy, but it undergoes no energy loss in a sample (in the form of heat and radiation), meaning a 100% conversion of energy resulting with no bleaching or heating effects, therefore allowing a long-term and high-powered observation of samples (Dempsey et al. 2012).

The response to the excitation wavelength depends on the polarization of a material interaction with light. For this, the total polarization, P, for a material interacting with light can be expressed as:

$$P = \chi^{(1)}E^{(1)} + \chi^{(2)}E^{(2)} \ldots + \chi^{(n)}E^{(n)} \tag{3}$$

The first term, where χ is the first-order nonlinear susceptibility tensor and E is the electric field vector, is correlated with linear interactions of light such as absorption, scattering, and reflection (Shen 1984). The second term describes SHG, sum, and difference of frequency generation; the third term describes two- or three-photon absorption, third harmonic generation (THG), and stimulated Raman processes and coherent anti-Stokes Raman scattering (CARS) (Chen et al. 2012).

The second-order symmetry nature of SHG enforces specific criteria on the available molecules that can be imaged using this property. Only molecules or regions lacking a center of symmetry (non-centrosymmetric) can be imaged. These molecules are known as harmonophores (Boddupalli and Bratlie 2015). Harmonophores and their environment must be non-centrosymmetric on the scale of the excitation wavelength being used. In addition, they need to possess a phase-matching condition, with the incident and resulting electromagnetic waves travelling at the same velocity as SHG is only efficient in coherent conditions (Vielreicher et al. 2013). Molecules being imaged must have a permanent dipole moment, and for further efficient SHG, they must be aligned within the focal volume of the microscope so that the second term of Eq. 3 is non-zero (Chen et al. 2012).

The principles of SHG facilitate the noninvasive imaging of structures such as type I and II collagen and myosin due to their distinctive molecular structure (Plotnikov et al. 2006; Campagnola 2011). Other isoforms of collagen do not meet these requirements because they lack a fibrillary organization in vivo; therefore, they do not produce sufficient SHG signal to be imaged (Pena et al. 2005).

4.2 Application of SHG in Biological Imaging

In biomedical applications, multiphoton SHG imaging has been demonstrated as an alternative to invasive microscopy involving tissue removal, fixation, and staining samples. Due to the structural and mechanical role of collagen and elastin in native tissue and tissue-engineered samples, simultaneous imaging of collagen and elastic fibers in tissue-engineered heart valves without resorting to standard histochemical protocols has been of particular interest. With this approach, noninvasive assessment of native tissue structures and quality control of engineered tissue has been achieved (Konig et al. 2005). Conventional ECM imaging requires fixation of samples and embedding in paraffin or cryopreservation and sectioning using microtomes prior to applying a specific staining, for example, picrosirius red (Lattouf et al. 2014). Although cryopreservation can allow storage of a tissue without fixation, rapid freezing can incur damage to the morphology of the tissue (Schenke-Layland et al. 2006). In contrast, paraffin-embedded tissue can maintain a reproducible representation of the tissue. By removing the paraffin and rehydrating the tissue, adequate multiphoton imaging can still be performed without staining protocols. Although if the sample was previously stained, even after following a destaining protocol, some interferences in the MPM/SHG signal will still exist (Monaghan et al. 2016).

Three major requirements to perform both multiphoton and SHG microscopy are a femtosecond mode-locked pulsed laser, a laser-scanning mode microscope, and bandpass filters that can separate SHG signal from multiphoton signal. One possible clinical approach is the acquisition of SHG using standard endoscopes or laparoscopes. Indeed Brown et al. have reported an endoscope capable of MPM/SHG imaging in vivo in unstained organs (Brown et al. 2012)3. Imaging of liver, kidney, and colon tissue revealed no significant differences in image quality between the use of an endoscope and a regular MPM/SHG microscope, and features commonly seen in biopsied histopathology slides from these tissues were observed (Brown et al. 2012). Although significant progress has been made in developing SHG technology for clinical applications, some areas are still undeveloped requiring further improvement and integration of all parts into a viable and easy-to-use instrument. Ultimately, multiphoton microscopy associated with SHG provides important details at submicrometer level of ECM and cells in vitro or in vivo while avoiding extensive sample preparation. Furthermore, hallmarks of disease progression, fiber alignment, and biomechanical properties can be assessed. By applying this noninvasive technique, it is possible to decrease surgical trauma, perform analysis in real time, and decrease sample manipulation. In addition, SHG can also be used to diagnose or follow treatment response in vivo by the use of an endoscope.

Fibrous collagen assembles into organized structures beginning in the nanometer range and then ordered into fibers (~50–500 nm) and then fascicles (~50–300 μm) (Puxkandl et al. 2002). A wave phase mismatch between the excitation source and the SHG emission can occur due to the quasicrystalline organization of collagen in biological samples (Prockop and Fertala 1998). This results in a separation of SHG emission into forward and backward components. The difference between both components is dependent on the regularity and the size of fiber assembly. Fibers

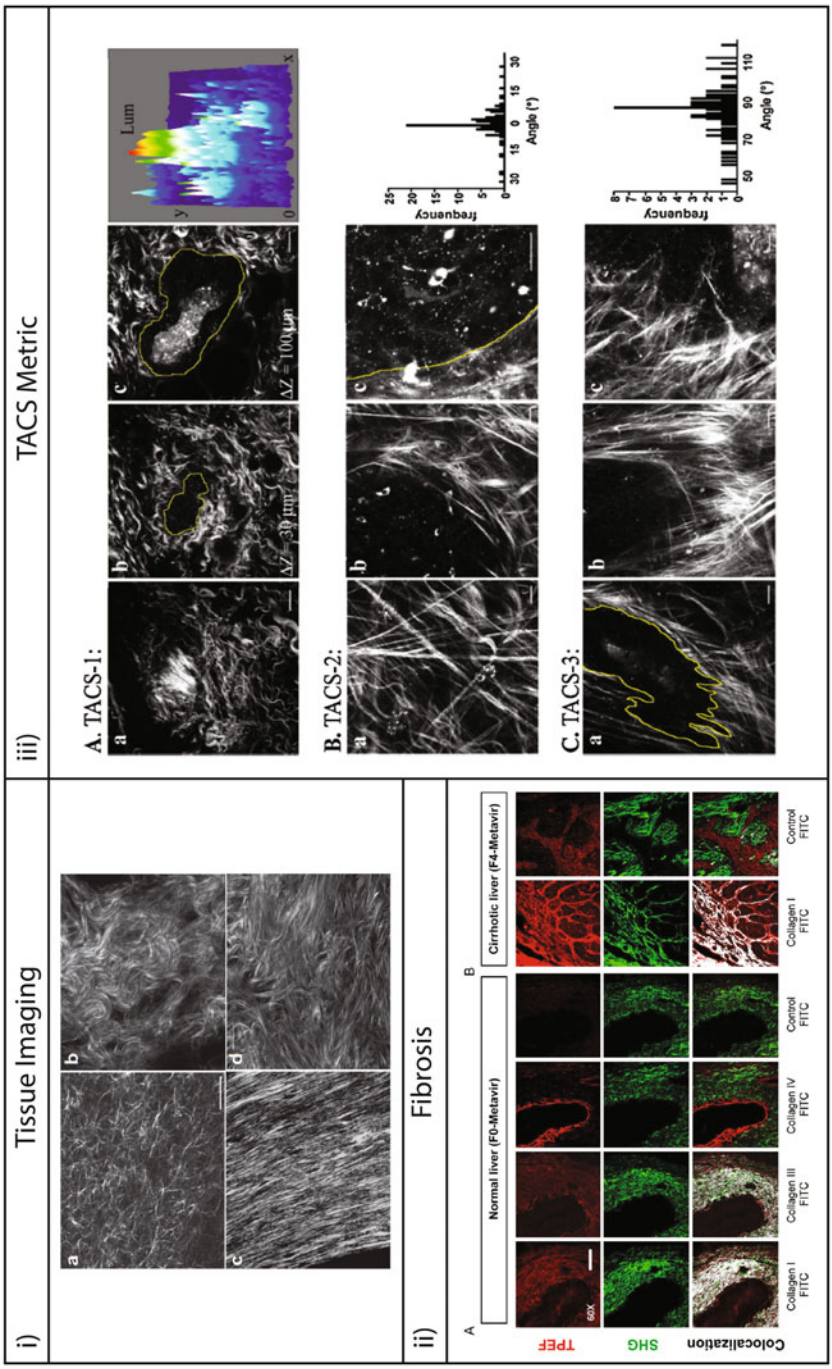


Fig. 3 (continued)

with an axial size in the same magnitude as the excitation wavelength will exhibit a predominantly forward-direction SHG signal, while fibrils with a smaller cross-section size produce equal levels of backward and forward SHG signals. This implies that for a comprehensive analysis of fiber alignment and collagen quantification, there is a need to capture both SHG signal scattering directions which would entail reflection and transmission paths with detection efficiencies calibrated to perform quantitative assessments.

4.3 Analysis of Pathologies Based on Fibrosis and ECM Remodeling Using SHG

SHG microscopy can facilitate the analysis of deposited collagen, thus allowing diagnosis of fibrotic development of internal organs or tissue (Strupler et al. 2007; Stanciu et al. 2014; Ranjit et al. 2016; Ochoa et al. 2018) (Fig. 3). The use of this technique is more advantageous over standard histology techniques: SHG allows the noninvasive acquisition of 3D image sets in in vivo or in vitro/ex vivo over thick samples (>10 μm) (Brown et al. 2003; Pantazis et al. 2010); and histology protocols can result in an erroneous staining of thin-sliced (1–10 μm) samples due to its effects on tissue morphology, given the rise to misguided interpretations (Strupler et al. 2007). Understanding collagen alignment is an important tool to evaluate disease progression, identification, and response to treatment. Nowadays, SHG is primarily employed as a research tool operated by highly trained researchers. However, using appropriate scoring systems will enable rapid tissue interpretation which will ease the application of this technique outside of the research field while reducing the training needed for new users.

Fibrosis is a common condition occurring during the development of chronic liver, kidney, heart, or lung diseases. It is characterized by an excess accumulation of freshly synthesized extracellular matrix proteins, such as collagen type I, impairing the normal function of the tissue resulting in more advanced and harder-to-treat pathologies (Bataller and Brenner 2005). The employment of SHG for fibrotic tissue assessment has been reported by Gailhouste et al. in the examination of human liver biopsies (both healthy and cirrhotic). These samples were classified using a

Fig. 3 SHG microscopy and applications. (i) Optical sections of collagen obtained in (a) self-assembled collagen; (b) mouse dermis; (c) mouse bone; (d) human ovary. Scale bar: 30 μm. (Reproduced from Chen et al. (2012) with permission from Springer Nature). (ii) Liver section immunolabeled and imaged by two-photon excited fluorescence (TPEF) and SHG in (A) healthy and (B) diseased liver. Unlabeled sections were used as control, and merging between both channels is displayed in white pseudocolor. (Reproduced from Gailhouste et al. (2010) with permission from Elsevier). (iii) Representation of the three TACS measures (A–C) in Wnt-I mouse tumors (a–c) showing tumor environment and correspondent boundary (yellow line). For (A) the intensity of the fluorescent signal relative to x-y location is present. For (B) and (C) histogram with the frequency distribution of fibers. Scale bars, 25 μm. (Reproduced from Provenzano et al. (2006) with permission from Springer Nature)

METAVIR fibrosis score ranging from F0 (no fibrosis) to F4 (cirrhotic tissue). With SHG, it was possible to formulate a fibrosis-SHG index which allowed for qualification and monitoring of different stages of disease progression while highlighting an easier, standardized methodology with lower sampling bias and able to correlate with the standard applied METAVIR fibrosis scale (Bedossa and Poynard 1996; Gailhouste et al. 2010) (Fig. 3ii). The use of SHG for analysis of fibro-proliferative pathologies is of higher interest for the medical field. Most of fibrotic evaluations need tissue obtained from biopsies. By developing a SHG scoring system (similar to fibrosis-SHG index), it is also possible to reduce the training needed to fruitfully apply this technique in a clinical environment.

Due to rapid cell division and high metabolic activity associated with tumor tissue, an excessive synthesis of ECM can lead to discernable differences in both composition and assembly of collagen in tumor tissue when compared with healthy tissue. This densely packed stroma has a negative impact on the diffuse transport of water and solutes due to the resistance conveyed by the interaction of collagen and proteoglycan components of the ECM resulting in a hindrance of drug treatment responses (Netti et al. 2000). Brown et al. reported the use of SHG in vivo which allowed dynamic imaging and quantification of collagen while rapidly inferring on different types of tumor susceptibility to drug delivery (Brown et al. 2003).

4.4 Biomechanics

The ultimate goal of tissue engineering is to repair and replace tissues and organs possessing distinct biomechanical functions which should be recapitulated. In addition to the tissue-specific biological requirements, there are criteria that functional engineered tissues should meet: size and mechanical integrity to allow survival under physiological conditions, minimal biomechanical functionality that should progress until the normal tissue function has been restored, maturation, and integration with surrounding host tissues (Robert Lanza 2014).

The alignment and organization of fibrillary collagen have an impact on the mechanical properties of the tissue. In this regard, SHG microscopy has been applied to assess and profile the biomechanics of cartilage (Lilledahl et al. 2011), arthritis disease models (Caetano-Lopes et al. 2009), arteries (Venkatasubramanian et al. 2010), the heart (Sommer et al. 2015), and the cornea (Winkler et al. 2011). The importance of collagen content and orientation for the native tissue function as well as the impact of poor collagen structure on organ function has been delineated using such techniques. The combination of multiphoton microscopy with mechanical stress testing can be also combined allowing a real-time analysis of collagen fiber orientation and alignment response to mechanical stress (Sereysky et al. 2012).

In addition to native tissue, SHG can also be applied to collagen-based (or SHG-responsive) scaffolds proposed for tissue engineering and biomaterial applications (Drury and Mooney 2003). Boddupalli et al. have applied SHG to evaluate collagen production from fibroblasts encapsulated in methacrylate alginate hydrogels. In softer gels, lower collagen production was observed, although their fibers were

more aligned in contrast to stiffer gels which promoted higher amounts of collagen with more anisotropic fibers. Both softer and stiffer gels may provide biomedical applications depending on the tissue of native collagen structure and alignment (Boddupalli and Bratlie 2019).

SHG is therefore an advanced microscopic technique that allows clinical, biomedical, and tissue engineering applications. With its increasing usage in research and weighing its features against more standardized procedures, it might be only a factor of developing a more user-friendly hardware, software, and easy-to-interpret results for SHG to be translated into the clinical field.

5 FLIM/PLIM Microscopy

Another advanced noninvasive imaging technology facilitated by multiphoton microscopy is fluorescence lifetime imaging microscopy (FLIM): a microscopy technique that generates an image based on the fluorescence intensity and on the spatial distribution of excited energy states lifetimes of an endogenous or exogenous molecule of interest in live or fixed cells and tissue. FLIM microscopes are designed to measure lifetimes from the nanosecond to microsecond (μs-FLIM or PLIM) range. Within cells there are molecules present with chemical configurations which enable them to undergo fluorescence emission after excitation with a specific UV/Vis wavelength. These components have within their molecular structure an aromatic group and a high number of π-bonds which increases fluorescence quantum yield and the probability of emitting light of higher intensity (Lakowicz 1999). The presence of fluorescent residues (tyrosine and tryptophan) in proteins is standardly measured in both enzymatic and biochemical assays for protein quantification (Stoscheck 1990).

Since the dawn of multiphoton technology, it has been possible to excite a cell's endogenous fluorophores in order to obtain fluorescence light emission by means of low-energy wavelengths that avoid phototoxic properties characteristic of higher-energy wavelengths (Diffey 1991). This emission is normally designated as cell autofluorescence. The molecules responsible for the autofluorescence emission are aromatic amino acids (tryptophan, tyrosine), serotonin, lipo-pigments (keratin, melanin), NAD(P)H, and FAD (see Table 1 for a more extensive list). In tissues, ECM molecules also contribute for the overall fluorescence of the sample mostly due to the presence of elastin and collagen and their high quantum yield which can be excited to perform SHG.

As described previously, the excitation of a fluorophore can be followed by emission of a photon, and depending on the radiative quantum yield of the fluorophore, this process can occur by fluorescence, phosphorescence, or delayed fluorescence. The predominant emission phenomenon measured in FLIM is fluorescence. The fluorescence lifetime of a molecule is the time – normally pico- to nanoseconds – that a fluorophore remains in an excited state before decaying and emitting a photon. Therefore, fluorophores can be characterized not only by their

Table 1 Examples of endogenous fluorophores commonly analyzed and used on FLIM

Fluorophores	Excitation peak (nm)	Emission peak (nm)	Fluorescent lifetime (ns)	Reference
NAD(P)H	690–730[a]	425–500	2.40 (long) 0.40 (short)	Alam et al. (2017)
FAD	890[a]	500–560	3.38 (long) 0.12 (short)	Alam et al. (2017)
Collagen	700–1000[a]	$\lambda_{ext}/2$	1.7 (long) 0.3 (short)	Dowling et al. (1998), Chen et al. (2012), Lutz et al. (2012), and Ranjit et al. (2016)
Elastin	700–1000[a]	400–650	1.95 (long) 0.26 (short)	Dowling et al. (1998) and Abraham and Hogg (2010)
Tryptophan	500–580[a] 710–730[b]	340–385	3.30 (long) 0.67 (short)	Jyothikumar et al. (2013)
Serotonin	560; 630	325–400	3.8	Botchway et al. (2008) and Chunqiang et al. (2010)
Keratin	720–800[a]	360–410	2.27 (long) 0.45 (short)	Ehlers et al. (2007), Dimitrow et al. (2009), and Chunqiang et al. (2010)
Melanin	800[a]	500–700	1.08 (long) 0.14 (short)	Dimitrow et al. (2009) and Chunqiang et al. (2010)
Tyrosine	565–610[a]	450–550	1.8	Lakowicz and Maliwal (1983), Kierdaszuk et al. (1995), and Shear et al. (1997)

[a]Corresponds to two-photon excitation
[b]Three-photon excitation and absence of marking are a standard single-photon excitation. Emission wavelengths (λemi) are indicated with correspondent fluorescence lifetime (τ) for double- and single-fluorescence decays

excitation and emission spectra but also by their unique lifetime. Fluorescence lifetime can be described by Eq. 4:

$$I_f = \alpha_1 \exp^{\frac{-t}{\tau_1}} + \alpha_2 \exp^{\frac{-t}{\tau_2}} + \ldots + \alpha_n \exp^{\frac{-t}{\tau_n}} \quad (4)$$

where $If(t)$ is the fluorescence intensity at a given time t and ūFC;$_n$ is the fraction of the fluorophore that is responsible for a specific τ_n fluorescence lifetime. The fluorescence lifetime decay of a fluorophore can be decomposed in several fractions depending on the molecule and its environment – mono-exponential or multi-exponential decay. If the fluorophore has a mono-exponential decay, ūFC;$_n$ value is 1, whereas in a multi-exponential decay, the sum of the various fractions ūFC;$_n$ is 1.

Fluorescence microscopy has been one of the most used tools in biological science, and it is mostly based on the fluorescent intensity of the specific fluorophores being analyzed. This technique is dependent on the light detection efficiency of the system and concentration of fluorophores and can result in variable levels of photobleaching and phototoxicity. Contrary to fluorescence microscopy, fluorescence lifetime is minimally affected by the aforementioned variables,

although it is sensitive to fluorophore microenvironment such as changes in pH, temperature, or presence of FRET donors/acceptors. Therefore, fluorescence lifetime measurements provide a more focused and profound analysis than measuring the fluorescence intensity (van Munster and Gadella 2005; Provenzano et al. 2009; Ishikawa-Ankerhold et al. 2012).

5.1 Frequency-Domain and Time-Domain FLIM

Acquisition of FLIM data can be performed in the frequency or time domains. Briefly, in frequency-domain FLIM, a high-frequency modulated laser is used to excite the specimen, and the fluorescence lifetime is determined by the demodulation and phase shift of the fluorescence signal (Schneider and Clegg 1997). Consequently, it is possible to determine the lifetime τ, from the measured phase shift and from the decrease in modulation from the emitted fluorescence compared with the excitation pulse. This will originate two separated lifetimes: τ_ω, lifetime calculated from the phase shift, and τ_A, the lifetime obtained from the modulation difference. If the fluorophore has a mono-exponential decay, $\tau_\omega = \tau_A$. For the analysis of more complex fluorescence lifetime decay patterns, measurements can be repeated using multiple modulation frequencies (Squire and Bastiaens 1999). To appropriately apply frequency-domain FLIM, it is required to have a modulated light source and to be able to extract both the phase and modulation signal from the excitation light and emitted light.

In time-domain FLIM, a short pulse of light is used to excite the sample after which the emitted fluorescence is measured in time resulting in curves described by Eq. 4 (Valentini et al. 2000). These measurements are acquired using a time-correlated single-photon counting (TCSPC) detector where after each excitation pulse, the arrival time of the first photon is monitored at a very high resolution (Phillips et al. 1985). A representative curve of the fluorescence lifetime decay is then obtained by recording the arrival time of the large number of photons being emitted by the sample. This time-domain technique is dependent on the detection sensitivity of the TCSPC unit to avoid bias toward photons with shorter lifetimes. Time-gated FLIM is another time-domain FLIM technique in which photons are collected at a fixed number in discrete intervals of time using a time-gated camera. The sample is excited with a short pulse of light, and the emission light is measured at two (or more) intervals of time during the fluorescence decay opposite to the previous technique where all the photons during a decay are measured. This way, the acquired lifetime is independent of laser intensity fluctuations. Increasing the number of time gates can provide more accurate results for multi-exponential decays (Sytsma et al. 2008).

FLIM measurements can be done in a nonspatially resolved fluorescence spectroscopy by exciting a sample or solution in a cuvette (Boens et al. 2007). For biomedical and tissue engineering fields, it is far more interesting to be able to use FLIM coupled with a microscope allowing morphological analysis and decomposition of complex samples by focusing the excitation volume or having the possibility

to 3D reconstruct a sample. Therefore, FLIM has been connected to wide-field (Gadella et al. 1994; Emiliani et al. 2003), confocal (Buurman et al. 1992; Buranachai et al. 2008), multiphoton microscopes (Gratton et al. 2003; Walsh et al. 2013), and light sheet microscopy (Mitchell et al. 2017; Funane et al. 2018)

Typically, the choice for the most adequate FLIM microscopy instrument is dependent on the specific application and spatial-time resolution besides financial consideration, equipment, and expertise available. Most of FLIM systems used in research have been custom-built by their users. Still, there are some considerations to be made between the different FLIM systems: wide-field and confocal microscopy are limited to one-photon excitation, while only confocal and multiphoton microscopy are capable of performing 3D FLIM z-stacks.

5.2 Fluorescence Lifetime Imaging Microscopy (FLIM) to Monitor Cellular Metabolism

All cells require a source of energy to maintain homeostasis and feed energy-consuming processes such as cytoskeletal dynamics, transcription, translation, and DNA repair. Cell metabolism also provides the building blocks for production of nucleotides, phospholipids, and amino acids (Lunt and Vander Heiden 2011). Metabolism in cells has evolved to sense locally available nutrient required to supply the cell to fulfil their biomass needs. When nutrients are scarce, cells halt biomolecular precursor production (amino acids, nucleotides, and acetyl-CoA) and adapt to extract maximum free energy from available resources to survive. Differences in metabolic pathways are dictated by distinct regulatory mechanisms that can control and adjust the cellular metabolism in differentiated and proliferative cells (Vander Heiden et al. 2009). In most mammalian cells, glucose and glutamine are predominantly catabolized and used as sources of carbon, nitrogen, and energy. The breakdown of glucose follows two major pathways: glycolysis and oxidative phosphorylation (OxPhos) to ensure efficient production of functional biomolecules and adenosine 5′-triphosphate (ATP) which is the main component of cellular energy and fuel for important homeostatic reactions happening in the cell (Fig. 4). Glutamine undergoes glutaminolysis which generates biomolecules and helps fuel the Krebs cycle (McKeehan 1982; Dang 2010).

Glycolysis is the starting point for the breakdown of glucose. It occurs in cell cytoplasm and culminates in the formation of pyruvate. From here, pyruvate is shuttled inside the mitochondria, aided by mitochondrial pyruvate carriers, or can be used as a substrate to produce lactate. In the mitochondria, the starting point of the OxPhos pathway, pyruvate is converted to acetyl-CoA which is used to feed the Krebs cycle by producing two electron carrier species (NADH and $FADH_2$) and important biomolecules for lipid, amino acid, and nucleotide synthesis, such as citrate, malate, oxaloacetate, and α-ketoglutarate. Afterward, both NADH and $FADH_2$ react with enzymes on the electron transport chain (ETC), specifically NADH/ubiquinone oxidoreductase (complex I) and succinate/ubiquinone reductase (complex II), respectively. Here, the passage of electrons between the different

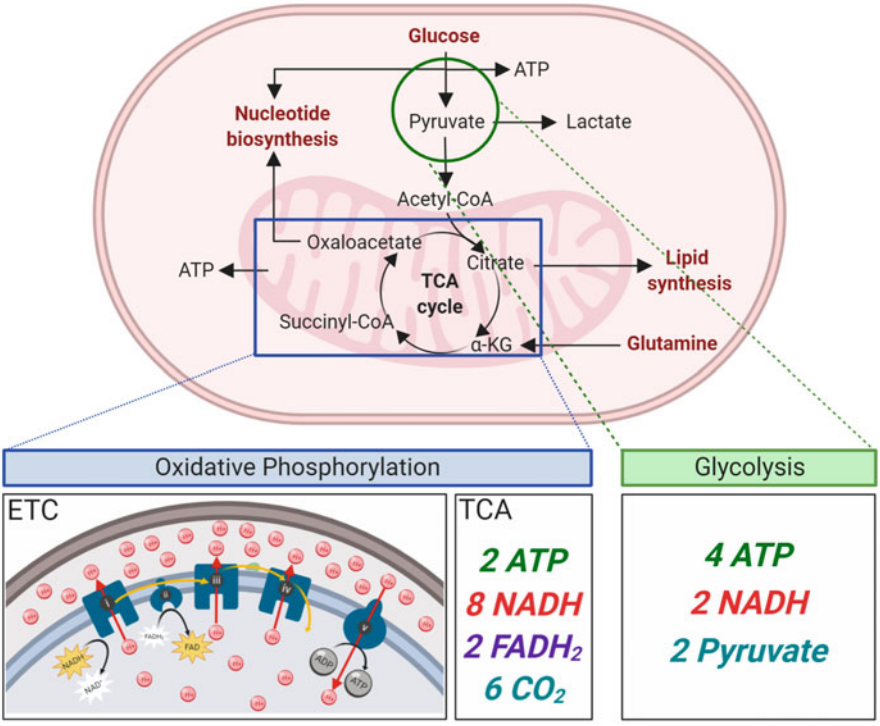


Fig. 4 Overview of glycolysis and oxidative phosphorylation. OxPhos was divided into two major pathways: tricarboxylic acid cycle (TCA) and the electron transport chain (ETC). Major by-products of glycolysis and TCA are highlighted as well as a schematic for the ETC process. (Figure was created with BioRender.com)

protein complexes releases energy which is stored as a proton gradient across the mitochondrial inner membrane ending in the formation of adenosine 5′-triphosphate (ATP) from adenosine 5′-diphosphate (ADP) at complex V or F_1F_0-ATPase. Overall, the OxPhos pathway yields 36 mol of ATP per mol of glucose consumed, and, in addition, it produces CO_2 and reactive oxygen species (ROS) while consuming O_2 (Fig. 4). In the cytoplasm, pyruvate conversion to lactate occurs without the consumption of O_2 and with only 4 mol of ATP produced per mol of glucose consumed (Folbergrová et al. 1974; Saraste 1999; Vander Heiden et al. 2009; Mookerjee et al. 2017).

Glutamine is imported to the cell and used for the production of glucosamine (in conjugation with glucose), nucleotides, and proteins. Furthermore, it undergoes glutaminolysis and is lysed by glutaminase into glutamate and ammonia. Glutamate is then imported into the mitochondria where glutamine dehydrogenase converts glutamate into α-ketoglutarate generating nicotinamide adenine dinucleotide phosphate (NADPH) while replenishing the Krebs cycle (Fig. 4). The last step of glutamine metabolism makes it an anaplerotic pathway which can be used to feed

oxidative phosphorylation if glucose concentration is low (McKeehan 1982; Dang 2010).

Aside from the availability of nutrients in the microenvironment, cellular metabolism can also be affected by other intrinsic or extrinsic factors such as genetic mutations (DeBerardinis et al. 2008), viral/bacterial infection (Thai et al. 2014), hormone or cytokine response (Bauer et al. 2004), cell density (inhibition by contact) (Aldridge and Pye 1976), or chemical metabolic challengers (Daemen et al. 2015). Interestingly, cell metabolism can be more than just a response to a stimuli or a microenvironment condition. An emerging field, known as immunometabolism, has established a definitive relationship between immune cell function and metabolism. It is widely acknowledged that M_1-polarized macrophages with pro-inflammatory responses are glycolytic, while anti-inflammatory M_2-activated macrophages are characterized by an oxidative phosphorylation-dependent metabolism (O'Neill et al. 2016). Not only is this metabolic preference observed in macrophages, but it is also exhibited in activated effector T cells and activated natural killer (NK) cells compared with their resting state (Zipfel et al. 2003b). In addition to immune cells, stem cells and differentiated cells possess distinct metabolic phenotypes. Stem cells are majorly glycolytic, whereas their differentiated counterpart is more OxPhos. Remarkably, when pluripotency is induced, their metabolic features revert back to a glycolytic state present in native stem cells (Folmes et al. 2012).

The coupling of FLIM measurements with multiphoton microscopy has opened a new modality to probe cellular metabolism in a noninvasive manner. Lakowicz et al. were the first to measure the fluorescence lifetime of free and protein-bound nicotinamide adenine dinucleotide (NADH) uncovering the potential of gauging this endogenous fluorophore and its impact on metabolism (Lakowicz et al. 1992). Since NADH has a single-photon excitation wavelength of 360 nm, it requires powerful and cell-damaging excitation sources. With the use of multiphoton microscopes, this problem is resolved by exciting NADH with 740–760 nm and allowing in vitro and in vivo metabolic profiling (Alfonso-Garcia et al. 2016; Niesner et al. 2004; Skala et al. 2007; Ghukasyan and Kao 2009; Yaseen et al. 2013). Following this, Huang et al. reported fluorescence spectroscopy and multiphoton microscopy of both NADH and FAD which are spectrally distinct at 750 and 900 nm excitation wavelengths. In addition, it is possible to separate both light-emitting species by their emission wavelength provided an adequate 410–490 nm bandpass filter for NADH and 510–560 nm bandpass filter for FAD (Huang et al. 2002).

With FLIM it is possible to uncover the role of these factors on the major cellular metabolic pathways (Fig. 4). Therefore, FLIM microscopy opens a pathway for noninvasive metabolic imaging and analysis that allows deeper understanding of the connections between metabolism and cell function or phenotype while enabling metabolic probing in real time. Furthermore, it can unveil distinctive metabolic features present in diseases (e.g., cancer), consequences of external stimulus (e.g., viral infection), as well as microenvironment nutrient concentration. By combining metabolic modulators (e.g., biomaterials or pathway inhibitors) and FLIM, it is possible to tailor and observe cell metabolism and infer on their impact and improve treatments, immune cell function, or stem cell differentiation while uncovering cell

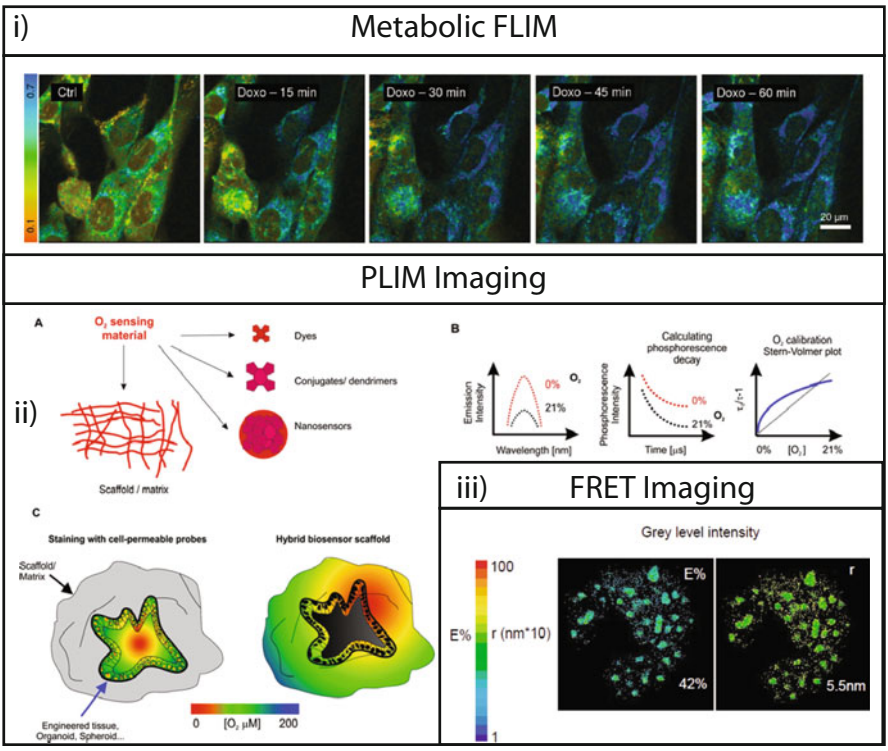


Fig. 5 Overview of FLIM, PLIM, and FRET imaging. (i) In vivo multiphoton FLIM images representing the continuous effect of doxorubicin on the optical redox ratio of human prostate cancer cell line. (Reproduced from Wallrabe et al. (2018) with permission from Springer Nature). (ii) Functionalization strategies for scaffolds/matrixes (a, c) and dynamics of O2 concentration measurements by PLIM (b). (iii) Intensity-based FRET with estimation of energy transfer efficiency (E%) and distance between probes ® used to identify C/EBPa (CCAAT/enhancer-binding protein a) dimerization. (Reproduced from Wallrabe and Periasamy (2005) with permission from Elsevier)

dependence on a specific metabolic profile. Using a multiphoton microscope (MPM) coupled with a fluorescence lifetime imaging microscopy (FLIM) or phosphorescence lifetime imaging microscopy (PLIM) detector, it is possible to evaluate NADH and FAD (Masters et al. 1997; Skala et al. 2007; Digman et al. 2008) and O_2 concentrations using exogenous probes (Kondrashina et al. 2012) (Fig. 5). An emerging application of such noninvasive microscopy is cell-specific metabolic analysis which has found applications in tumor biology, immunology, and organoid research fields (Skala et al. 2007; Wallrabe et al. 2018).

NADH fluorescence lifetime typically exhibits a double-exponential decay which is decomposed in a short lifetime related to free NADH in the cytoplasm and a long lifetime reflective of enzyme-bound NADH (Lakowicz et al. 1992). Following Eq. 5 is possible to calculate the fraction respective to each of them. In a single-cell type, the enzymes in which NADH reacts are well-conserved regions in a cell, meaning

that the majority of the shifts in fluorescence lifetime fractions are connected with the free NADH fraction. Generally, when this fraction increases, the cell metabolic profile is tending toward glycolysis. Conversely, when this fraction decreases, the cell is less dependent on glycolysis (Schaefer et al. 2019). Regarding NADH fluorescence intensity, it has been shown that increasing glucose concentration in the cell culture medium or adding glycolysis inhibitors increases NADH autofluorescence, revealing a higher usage of the OxPhos pathway (Ghukasyan and Kao 2009). However, NADH fluorescence intensity and lifetime have limited capacity for fully grasping the cell metabolic profile; nonetheless, they are well suited for separating distinct cellular populations or following disease progression (Pugh et al. 2013).

The coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) possesses an overlapping excitation and emission spectra with NADH and is hard to be separated (Blacker and Duchen 2016). Although the NADPH contribution is ten times lower than NADH, NADPH still contributes significantly to cell autofluorescence (Blacker et al. 2014). Fluctuations of NADPH levels are intertwined with the long-lived component of the fluorescence lifetime decay – protein-bound NADH. NADPH processes two major biological functions: acting as an electron source for synthesis of fatty acids, steroids, and DNA and a key component in cellular antioxidant systems intertwined with the production of reactive oxygen species (Pollak et al. 2007; Ying 2008). It is still important to understand how the metabolic profile is being affected in the experimental conditions or to perform a biochemical assay to fully understand the impact of NADPH on the cell metabolism.

Assessing other metabolic cofactors besides NADH can add additional information to metabolic profiling. The endogenous molecule flavin adenine dinucleotide (FAD) is commonly used due its autofluorescence. After measuring the fluorescence intensity of both cofactors, it is possible to calculate the redox ratio by following Eq. 5:

$$\text{Redox Ratio} = \frac{FAD_{\text{photons}}}{NADH_{\text{photons}}} \tag{5}$$

This redox ratio has been associated qualitatively with metabolic profiling after challenging of the glycolytic or oxidative phosphorylation pathway (Walsh et al. 2013). During both these metabolic pathways, there is a regeneration of fluorescent NADH, and during OxPhos, the formation of non-fluorescent $FADH_2$. Following Fig. 4, NADH is converted to non-fluorescent NAD^+ by complex I and $FADH_2$ to fluorescent FAD+ by complex II of the electron transport chain. When OxPhos is more active, there is more production of $FADH_2$, and therefore more FAD+ production leads to a rise of FAD^+ fluorescent intensity culminating in an increase of the redox ratio. On the contrary, when glycolysis is majorly used to obtain energy, NADH is produced increasing its fluorescence emission, and FAD^+ regeneration has been hindered resulting in a decrease of the redox ratio (Ostrander et al. 2010). Furthermore, it is possible to combine both fluorescence intensity measurements and lifetimes to originate the optical redox ratio (ORR) which confirms and better describes the shifts in cellular metabolism (Fig. 5i) (Walsh et al. 2012).

While measuring endogenous NADH/FAD (redox or optical metabolic imaging) is highly informative, frequently there is a need in assessing additional parameters of cell metabolism, such as analysis of mitochondrial polarization, oxygen consumption, extracellular acidification, lipid droplet production, and others. The majority of these additional parameters are measured via exogenously introduced or genetically encoded fluoro- and phosphores (Dmitriev 2017).

For example, a number of genetically encoded fluorescent protein biosensors (which require transfection to be introduced into eukaryotic cells) can serve as indicators of redox, peroxide production, or NADH/NAD^+ ratio (Mongeon et al. 2016; O'Donnell and Dmitriev 2017). Mitochondrial membrane potential can be assessed by intensity- or FLIM-based measurement, with the help of tetramethylrhodamine methyl ester (TMRM) dye (Brand and Nicholls 2011; Okkelman et al. 2019b). TMRM is a membrane-permeable molecule which accumulates mainly in both the inner mitochondrial membrane and matrix space of live cells due to its charge and solubility. The change in TMRM uptake is linked to shifts in the mitochondria membrane potential which results in variations of the fluorescence intensity. By measuring the fluorescence intensity at 573 and 546 nm, it is possible to estimate the mitochondria membrane potential (Scaduto and Grotyohann 1999). This molecule also has the possibility to be used as an exogenous multiphoton FLIM probe (Okkelman et al. 2019b).

In addition to endogenous or exogenous fluorophores, there is also the possibility to employ genetically encoded fluorescent biosensors. One strategy to create these protein biosensors involves using circularly permuted fluorescent proteins derived from GFPs (Baird et al. 1999). The circular permutation joins the original N and C terminal by a peptide linker, and a specific reporter or binding protein is fused to the new coupled terminal complex originating a conformational coupling between fluorescence emission and binding to a molecule of interest (Hung et al. 2011). This approach has been applied to probe further the glycolysis flux (Shestov et al. 2014), intracellular pH (Tantama et al. 2011), NADH/NAD^+ (Masia et al. 2018), and ATP/ADP ratio (Berg et al. 2009).

To further evaluate cell metabolism, an important measurement to be done is oxygen consumption rate (OCR) and/or analysis of relative tissue oxygenation, balanced by oxygen consumption and diffusion. This can be performed by using a number of analytical tools, ranging from labelling with pimonidazole and use of fluorescent proteins to phosphorescence or delayed fluorescence-based approaches (Papkovsky and Dmitriev 2018). Quenched phosphorescence-based detection by using phosphorescence lifetime imaging microscopy (PLIM) is among the popular approaches in tissue engineering, and intravital microscopy and will be discussed later in this chapter (Sect. 5.4).

When performing live-cell imaging, it is necessary to consider possible contributions of the fluorophores described in Table 1, especially where some tissues have fluorophores in abundance such as the brain (serotonin) and epidermal (keratin, melanin) tissue. Nonetheless, by careful selection of excitation wavelengths and bandpass filters, it is possible to ascertain the contribution of each molecule (Huang et al. 2002).

5.3 FLIM-FRET

In the cellular environment, signaling molecules, enzymes, proteins, and other cellular components undergo many dynamic process and interactions that occur in a microsecond or a nanosecond range. Due to the interest in understanding complex cellular pathways with high spatial and temporal specificity, sophisticated imaging technologies such as one- or two-photon Förster resonance energy transfer (FRET) imaging have been developed (Wallrabe and Periasamy 2005).

This imaging technique operates on the transfer of energy (radiative or non-radiative) between a pair of suitable fluorophores ("donors" and "acceptors") in close proximity (2–10 nm) (Ranjit et al. 2016). For this to occur, both molecules must have sufficient spectral overlap, a favorable dipole-dipole orientation, and a large quantum yield. During the energy transfer process, the donor fluorescence decreases (quenched), and the fluorescence of the acceptor increases, culminating in a decrease in the donor excitation lifetime.

The energy transfer efficiency between a donor and acceptor can be assessed using a confocal or a multiphoton microscopy in fluorescence intensity FRET approaches. Two major approaches of FRET can be used in biomedical research: the first one – qualitative FRET – is used for establishment of molecular colocalization, cellular organization, or conformational changes, where the presence or lack of FRET is a sufficient indicator for the experimental plan. Panyi et al. utilized FRET to observe the presence and colocalization of potassium channels and CD3 molecules in human T lymphocytes by evaluation of fluorescence intensity and selective light emission (Panyi et al. 2003). The second approach to FRET is known as quantitative FRET. Here, values such as the energy transfer efficiency (E%) and distance between fluorophores are estimated. This method was used by Rinnenthal et al. to quantify intracellular Ca^{2+} in in vivo and ex vivo studies and its relationship with neuronal dysfunction during neuroinflammation (Fig. 5iii) (Rinnenthal et al. 2013).

The spectral overlap needed for an efficient FRET process is also the main cause of experimental errors (Sekar and Periasamy 2003). As it possesses risk of signal contamination, some corrections may have to be performed. The main reason for signal contamination is the spectral overlap between both molecules necessary for FRET to occur, and this is a major pitfall of conventional FRET imaging. To avoid these corrections, FLIM can be applied to measure FRET as it takes in account the change in a donor lifetime in the presence and in the absence of an acceptor (Elangovan et al. 2003; Wallrabe et al. 2003), in contrast to intensity-only-based measurements.

Using FLIM-FRET, Lamond et al. presented a quantitative platform to measure chromatin compaction in live cells (Lleres et al. 2009). In this study, cells genetically engineered to coexpress histone H2B tagged to an enhanced green fluorescence protein or mCherry were used. The FRET process occurred between separate nucleosomes and increased as chromatin became more compact. In the same cell population, varying FRET efficiency levels were observed reflecting distinct compaction levels, spatially distributed.

To summarize, FLIM-FRET microscopy allows real-time imaging with a high resolution (spatial and temporal) of molecular interactions. It has been applied to investigate several biological processes such as signaling pathways, conformational changes, or binding molecules. The use of this technique is increasing with technological advances such as detectors, microscopes, or fluorophores. One interesting advance is the use of quantum dots which could improve both quantitative and qualitative FRET; however, cell toxicity and cell delivery issues need to be solved.

5.4 Measurement of Molecular Oxygen (O2) in Tissue-Engineered Constructs by PLIM and Related Approaches

Since the oxygen-dependent quenching of fluorescence was first described by Kautsky and Hirsch in 1935 (Kautsky and Hirsch 1935), this methodology has evolved to be a popular approach to measure vascular and brain oxygenation; analyze hypoxia in cells, tissues, and aquatic organisms; and study enzyme activity, microbial growth and is exploited in many other areas (Sakadžić et al. 2010; Papkovsky and Dmitriev 2013; Roussakis et al. 2015; Wolfbeis 2015; Yoshihara et al. 2017; Dmitriev and Papkovsky 2018). This methodology is based on the specific collisional quenching of dye phosphorescence (frequently encountered with metalloporphyrins and related metal-coordinated organic complexes) by molecular oxygen, resulting in quantitative decreases of emission intensity and lifetime (typically from 1–2 to hundreds of microseconds). In tissue engineering, the dye can be modified and used in two ways (Fig. 5ii):

1. Become a molecular or supramolecular probe or impregnated in nanoparticles. In this case, the probe or nanosensor has to be delivered into the cell or tissue-engineered construct (multicellular spheroids, islets, or organoids).
2. Impregnate phosphorescent O_2 dyes within matrices or scaffold materials surrounding, or in contact with, live tissue – "hybrid scaffold biosensor" approach (see below).

Due to long emission lifetimes and large Stokes shift, phosphorescent probes are highly compatible for multiplexing in spectral and time domains, e.g., with other FLIM probes, antibodies, conventional fluorescent dyes, and protein biosensors. In addition, on a technical level, PLIM is easier to grasp, and due to long emission lifetimes, even microsecond time-gated cameras can be used to perform measurements (Dmitriev et al. 2013). Presently, PLIM is also available on a TCSPC-FLIM and, in frequency modulation-based platforms, with wide-field, confocal, and two-photon excitation modes.

The "hybrid biosensor scaffold" approach enables detection of O_2 in proximity of the live tissue, without the need to stain tissue or cells. With high respiratory activity primarily characteristic of live cells and 3D tissue models, the scaffold-based sensing of O_2 is very informative and can be used to predict the hypoxia-dependent drug action within engineered tissue, cell differentiation, and wound healing or simply

ascertaining the availability of oxygen to the tissue. This approach normally uses electrospinning coating-aided impregnation of dyes or their impregnation in polymer structures due to swelling in organic solvents (Xue et al. 2014, 2015; Jenkins et al. 2015; Yazgan et al. 2017; Roussakis et al. 2019; Schilling et al. 2019). For instance, porous polystyrene-based O_2-sensing scaffolds doped with highly photostable PtTFPP O_2-sensing dye have demonstrated high compatibility (lack of direct and phototoxic effects and reliable performance in quantitative measurements) with cancer cells and live brain slices and multiplexing with mapping of cell death and mitochondria labelling (Jenkins et al. 2015). Recent work has demonstrated the dual use of impregnated phosphor in bone regeneration process, both for two-photon O_2 sensing inside the scaffold and within the area surrounding it, achieved by predictable diffusion/leaching of dendrimeric phosphor from the scaffold (Schilling et al. 2019). It is worth noting that since the amount of the impregnated/scaffold-associated phosphorescent dye can be controlled and remains unchanged during the experiments, the simple fluorescence intensity-based calibration and sensing can be used as alternative to PLIM measurements.

In many applications, researchers strive to measure O_2 directly inside cells, and a great variety of intracellular O_2 probes, nanosensors, or even dye structures are available for this purpose (Dmitriev and Papkovsky 2015). However, the majority of engineered tissue constructs are three-dimensional which poses a challenge for diffusion-limited delivery of any probe or nanosensor and for imaging, due to limits of light penetration/absorption and scattering. A priori, the best approach is to employ two-photon microscopy although laser scanning confocal or even wide-field imaging of relatively small engineered tissue samples (50~300 μm) can result in successful imaging. Multicellular spheroids are among the most popular models for engineering tissue environment in 3D and were the first model to demonstrate challenges in probe staining for O_2 and fluorescence imaging (Dmitriev et al. 2013). These studies reported the multiparameter PLIM of stem cell-derived neurospheres combined with analysis of distribution of hypoxia-specific dyes, cell proliferation, and differentiation markers by immunofluorescence (Dmitriev et al. 2013, 2014). Further on, multiplexed FLIM-PLIM was reported with tumor spheroid and small intestinal organoid models for measurements of oxygenation together with analysis of cell proliferation, temperature imaging, and biosensor scaffold-based analysis of extracellular acidification (Jenkins et al. 2016; Okkelman et al. 2016, 2017; O'Donnell et al. 2018). In principle, O_2-PLIM is also compatible with NAD(P)H-FLIM, which was demonstrated by the Rueck group (Kalinina et al. 2016), but only recently has combining NAD(P)H-FLIM with O2-PLIM been applied to analyse tissue-engineered constructs (Okkelman et al. 2019a).

Collectively, PLIM brings advantage of measuring tissue oxygenation in 3D setting, by means of scaffold- and cell staining probe-aided sensing modalities. While the method is still in its infancy in respect to tissue engineering and its advantages are yet to be fully realized, it has already been successfully used in proof-of-concept studies with tumor and stem cell-derived spheroids, organoids, and implanted tissue models. Its emerging applications in tissue engineering are in the multiplexed analysis of cell bioenergetics, control of tissue viability, and studies of therapeutic outcomes under controlled biomaterial oxygenation.

6 Intravital Microscopy (IVM)

Intravital microscopy (IVM) is a powerful optical imaging technique that facilitates continuous monitoring of molecular and cellular processes in vivo. The main principle of IVM is to image and collect information of biomolecular processes occurring in tissue and organ systems while maintaining their native and physiological state. However, accessing the in vivo environment and performing deep-tissue imaging are incompatible with standard microscopic techniques due to light absorbance and scattering. In order to improve tissue penetration, IVM is mostly performed using red or near-infrared excitation wavelengths. In addition, new optics and more flexible instruments are being developed to improve internal organ access (Bullen 2008). To perform quantitative IVM, it is necessary to consider four major components: site preparation for visualization, use of an endogenous or exogenous probe that can be detected by microscopy, a microscope mounted with appropriate detectors, and computer algorithms and mathematical models that can be used to obtain important parameters. Regarding tissue preparation, IVM has three major categories: fitting chronic-transparent windows at the site of interest in the animal model, exteriorized tissue preparations, and in situ preparations (Jain et al. 2002). Most common animal models preferentially subjected to IVM are murine due to its systemic dynamics and zebrafish due to its small size, ex utero development, transparent embryos, and availability to have transparent adult mutants. Transgenic versions of these animal models can also be prepared in which encoding fluorescence proteins in cell lineages or proteins of interest are considered the ideal tools to perform IVM (Progatzky et al. 2013).

One of the major developments in IVM microscopy has been the application of multiphoton microscopy. MPT allows higher resolution and increased tissue penetration, without compromising fluorophore selection, less photodamage and phototoxicity, and better signal-to-noise ratios (Helmchen and Denk 2005; Molitoris and Sandoval 2005; Misgeld and Kerschensteiner 2006; Bullen 2008). However, the cost and the complexity of setting up, adapting to IVM, and maintaining a multiphoton microscopy system has been a barrier to its wider implementation (Zipfel et al. 2003b). Nonetheless, the major advantage of IVM is the possibility to image and analyze pathologies, perform cell tracking, and measure gene or protein expression in real time on a full systemic environment in long-term studies without needing to excise tissue. In addition, IVM can be easily incorporated into other microscopy methods.

6.1 IVM Coupled to Confocal Microscopy

Nomoto et al. used real-time intravital confocal microscopy to evaluate quantitatively polyplexes and polyplex micelles present in the blood circulation with the ultimate goal of uncovering the interaction between non-viral gene vectors and biological components in situ. To achieve this, Cy5 fluorophore was used to label plasmid DNA within polymeric complexes and DyLight 488 conjugated to an anti-GPIbβ to label platelets (Nomoto et al. 2011). Interestingly, another study used

confocal microscopy to evaluate platelet dynamics in response to inflammation. Here they evaluated in real time LPS-induced inflammation in phycoerythrin-conjugated CD49b antibody-labelled platelets on the liver, brain, and muscle tissue (Jenne et al. 2011). These examples showcase the ability of using confocal microscopy allied with IVM. Although there are some known limitations of confocal microscopy in terms of tissue penetration, the use of red and far-red fluorophores coupled with antibodies to label specific cells makes it possible to perform cell tracking in regard to distribution, size, and activation in response to internal or external stimulus in different tissues.

6.2 IVM Coupled to Multiphoton Microscopy

Multiphoton microscopy has made it possible to perform deep tissue imaging in animal models for extended durations. It allows the use of endogenous or exogenous fluorophores for specific cell tracking. One field that benefited immensely from this coupling is immunology. Indeed, many important immune cell behaviors occur in densely populated tissues, and it was previously only possible to delineate these activities in vitro. Several studies document the reliability to infer immune cell function in vivo using multiphoton IVM (Mempel et al. 2004; Cavanagh and Weninger 2008; Egen et al. 2008; Hickman et al. 2008; Abtin et al. 2014; Marques et al. 2015). For example, Hickman et al. imaged CD8+ T-cell function in virus-infected cells in the peripheral interfollicular region of lymph nodes. Here, fluorescein isothiocyanate (FITC) and fluorescein-conjugated dextran (500 kilodaltons) were injected to image the lymph node subcapsular sinus and blood vessels, respectively. In addition, complexes of fluorescein-conjugated dextran (70 kilodaltons) and FITC-conjugated antibodies were used to label macrophages and areas of interest (Hickman et al. 2008). This facilitated the identification of virally infected cells, naïve T-cell interaction and activation by dendritic cells in real time which underlined T-cell effector function with dendritic cells, and antiviral response in lymph node periphery. Abtin et al. also uncovered interactions between different immune cell populations in response to a bacterial infection (Abtin et al. 2014). In virally infected tissues, perivascular macrophages release neutrophil chemoattractant signaling molecules promoting the recruitment of neutrophils to the damaged area which was followed in transendothelial and intravascular regions (Abtin et al. 2014). Genetically engineered mice expressing enhanced green fluorescence protein (EGFP), yellow fluorescence protein (YFP), and green fluorescence protein (GFP) were used to track immune cell subpopulations. Evans blue stain and *S. aureus* expressing red fluorescent protein (RFP were used to identify blood vessels and bacteria inside of the tissues. In addition, SHG was also used to improve epithelial tissue and blood vessel identification by visualization of both collagen and elastin (Abtin et al. 2014). These two studies present an interesting application of multiphoton IVM in immunology field which allowed cell-specific tracking and function inside of tissues and blood vessels on an in vivo and systemic approach.

6.3 IVM Coupled to FLIM

The use of FLIM with IVM synergizes the benefits of multiphoton microscopy while analyzing only endogenous fluorophores such as NADH or FAD which reduces sample manipulation and preparation time. This allows to infer also the in vivo metabolism of specific cell types and tissues in real time (Rinnenthal et al. 2013; Thorling et al. 2013; Hato et al. 2017). Hato et al. performed intravital two-photon FLIM on mouse kidney revealing specific metabolic signatures of different cells. Using FLIM revealed significantly different metabolic profiles of S1 and S2 tubules. However, these differences may have been due to microenvironmental factors such as pH, calcium, and oxygen levels. Interestingly, similar metabolic profiles were uncovered between S1 proximal tubules and distal tubes; therefore, these two segments may share similar metabolites and microenvironments. In addition, by combining two-photon FLIM- IVM on genetically engineered mice expressing DSred in podocytes and endothelial-specific CreERT2 displaying red fluorescence in the endothelium, elusive peritubular capillary endothelium and podocytes were imaged (Hato et al. 2017). This study serves as an example of IVM-FLIM application to image entire or specific healthy and diseased regions in a tissue with subcellular resolution allowing to metabolically profile areas and specific cell types.

6.4 Förster Resonance Energy Transfer (FRET) in IVM

Biomolecular dynamics can also be probed with IVM and FRET. Most FRET (and FLIM-FRET) methods are still applied in vitro. However, in order to achieve an increasing understanding of molecular processes and cellular function in vivo, it could be worthwhile to apply this technique to a highly complex and functional organism (Radbruch et al. 2015). There has been a surge in strategies to incorporate FRET probes within living organisms. One approach is the development of transgenic mice which encode and express fluorescent FRET biosensors, allowing for long-term monitoring of cell and tissue function in healthy and disease models (Thestrup et al. 2014). An alternative and quicker method is lenti- or retroviral transfection of cells in vitro and then transplantation into a living organism (Breart et al. 2008). The main advantage of transplanting genetically modified cells compared to the use of transgenic animal models is the ability to characterize the cells and the FRET biosensors in vitro prior to transplantation (Timpson et al. 2011). Janssen et al. applied IVM-FRET to evaluate tumor cell viability and mitosis during chemotherapy treatment. In this study, the mechanism of action of microtubule-targeting chemotherapeutics belonging to a class of pharmacological molecules called taxanes was compared in both in vitro and in vivo applications. In in vitro studies, the major effect of the treatment observed was induction of apoptosis by mitotic cell death which was previously hypothesized to occur in vivo. However, in vivo the majority of tumor cells died independently of mitotic defects, highlighting a different mechanism occurring in a systemic environment. To observe this difference, caspase-3, a crucial protease for apoptosis induction, was modified with cyan

fluorescence protein (CFP) and yellow fluorescent protein (YFP). When caspase-3 is inactive, both fluorophore domains are in close proximity, and, as a result, CFP fluorescence emission decreases as it is transferred to YFP which in turn increases its emission leading to a low CFP to YFP emission ratio. When caspase-3 is activated, a specific motif is cleaved, separating CFP and YFP, rendering a high CFP to YFP ratio (Janssen et al. 2013). This approach illustrates the power of coupling FRET to IVM in order to evaluate biomolecular processes in vivo while comparing to in vitro results.

7 IVM Cell Tracking Studies

IVM can facilitate in vivo study of cellular and molecular dynamics that are limited by in vitro conditions. To fully understand the impact of diseases and drug development in cell biology, there is a need to ensure clinical translatable results and its effect on a complex organism. Tumor biology and immunology are two major fields in which the application of IVM can deepen the understanding of in vivo cellular and molecular processes. Here, labelling cells with exogenous fluorophores or by using endogenous fluorescent molecules provides a window to follow in real time specific cell types, tumor and metastasis development, responses to treatment, and reactions to bacterial/viral infection and cell migration (Fig. 6).

7.1 Cell Division and Cell Death

Tumor cells are characterized by their ability to undergo and sustain proliferative behavior. Labelling multiple intracellular components can enable the monitoring of mitosis and apoptosis. One approach considers the use of fluorescence tags for histone H2b and α-tubulin, allowing the nuclear morphology to be observed. Orth et al. by using this method demonstrated that cell division occurs similarly in both in vivo and in vitro conditions; however, in vivo it appears to be a far less frequent event (Orth et al. 2011). Several approaches to evaluate cell death in IVM are available. One tactic is a readout based on a red cytoplasmic (CMTMR) and a blue nucleic (Hoechst33342) dye. In this approach, as a cell undergoes apoptosis, there is a measurable variation in the cytoplasm-to-nuclei fluorescent ratio due to the loss of soluble proteins from the cytoplasm and an increase of emission as the nuclei becomes apoptotic (Mempel et al. 2006). Other methods employ FRET fluorophores to follow the triggering of cell death mechanism such as caspase activity or p53 DNA damage. This is an interesting application of IVM to understand autophagy, apoptotic pathways, and response to drug treatments or to follow a certain pathology cycle (Breart et al. 2008; Spencer et al. 2009; Giesen et al. 2011; Lawson et al. 2015) (Fig. 6i). For specific tracking of dividing cells or cell death, IVM on its own, or coupled with FRET, provides multiple approaches that can be used by researchers to understand cell responses to an internal or external stimuli impacting cell fate.

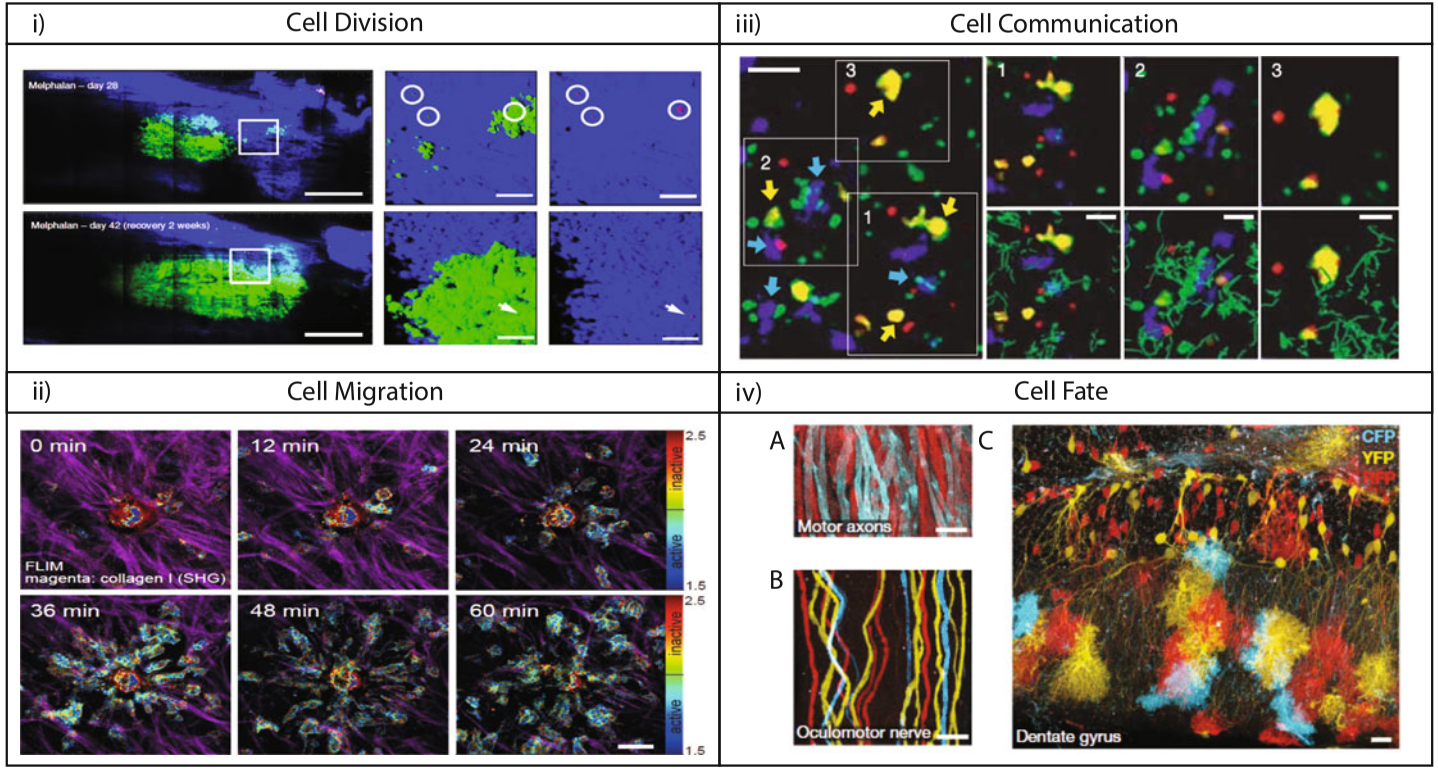


Fig. 6 IVM cell tracking in vivo. (i) Mosaic image of myeloma cell division revealing resistance to chemotherapy drugs triggered by highlighted (white circles and arrow) and red cell activation. Scale bar: 500 μm (left) and 100 μm (middle and right). (Reproduced from Lawson et al. (2015) with permission from Nature Publishing Group) (ii) Time-lapse image of neutrophil attraction to site of laser damage in vivo. Scale bar: 25 μm. (Reproduced from Nobis et al. (2017) with

7.2 Cell Migration

Cell mobility is an important feature of cancer invasiveness and immunological responses. This movement is dependent on adhesion, mechanosensing, and cytoskeletal remodeling. With IVM it is possible to image at defined time intervals and follow cell migratory paths, recruitment, velocity, and interaction with other tissue components (Sumen et al. 2004; Nobis et al. 2017). Applying this approach has added a deeper understanding of cell movement in vivo compared to limited in vitro studies. Interestingly, leukocyte movement in vivo relies primarily on the expansion of the actin network and without specific interaction within the extracellular environment; however the in vitro migration is dependent on additional factors such as the anchoring of the cellular membrane by integrins (Lammermann et al. 2008); depending on the cell type, movement is also triggered by chemokine receptor-ligand communications (Okada and Cyster 2007); cells can migrate as one cohesive group primarily due to their cell-cell junctions in a process known as "collective cell migration" (Alexander et al. 2008) or "cell jamming" (Haeger et al. 2014). Furthermore, this technique has been applied to follow both adaptive and innate mobilization of immune cells to inflammation areas (Fig. 6ii) (Auffray et al. 2007; Egen et al. 2011). Applying IVM to survey cell migration in vivo takes in account factors difficult to replicate in in vitro conditions. 3D environments, interaction with soluble factors, and cell and systemic signaling are continuously present in dynamic in vivo environments.

7.3 Cell Communication

Communication between different cells types is established either by chemical signaling, such as the release of cytokines or by physical interactions. Such signaling has an effect on cell proliferation, survival, mobility, and activation of effector functions (Mantovani and Dejana 1989; Goldring and Goldring 1990). The application of IVM to cell-cell interactions is still in its infancy. This is mostly due to the restriction of tracking whole cells, organelles, and macromolecular structures instead of their molecular activities related with production, secretion, and response to effector molecules. Therefore, there is a need to complement current IVM

Fig. 6 (continued) permission from Elsevier). (iii) Imaging of cellular contacts between CD4þ T cells (red) and polyclonal CD8þ T cells (green) mediated by pulsed dendritic cell (blue/blue arrows) and medium-pulsed dendritic cells (yellow/yellow arrows). The top row of the right panel shows enlarger views, while the bottom row shows the movement (green) made by CD8þ T cells. Scale bar: left, 30 mm; right, 15 mm. (Reproduced from Castellino et al. (2006) with permission from Springer Nature) (iv) Brainbow expression in genetically engineered mice promote expression of (A) M-CFP in peripheral motor axons and multiple fluorescence proteins in (B) oculomotor nerve and (C) hippocampus (dentate gyrus). (Reproduced from Livet et al. (2007) with permission from Springer Nature)

cell-communication experiments with ex vivo studies. Nonetheless, IVM has been used to showcase the complexity of cellular communication and their molecular microenvironment in vivo.

IVM has demonstrated interactions that boost or suppress adaptive immune responses. Here, symbiotic interactions between dendritic cells and CD4 helper T cells result in the release of chemokine signals that promote migration of CCR5+ CD8 T cells (Fig. 6iii) (Castellino et al. 2006). Another IVM cell tracking study has demonstrated that communication between dendritic cells and CD4 regulatory T cells actually suppresses CD4 helper T-cell actions (Tang et al. 2006). In tumor biology, cell communication has been found to have an important role in aggressive tumor microenvironments. It has been showed that perivascular macrophages interact with cancer cells and are hijacked to facilitate cancer cell migration and entry into lymphatic or blood vessels (Wyckoff et al. 2004). SHG coupled with IVM has found that migration of macrophages and tumor cells occurs primarily along collagen fibers which exemplifies the importance of extracellular matrix alignment and density to invasive tumor microenvironment (Roussos et al. 2011). In addition, premetastatic circulating tumor cells have been showed to halt at vascular branch points where they infiltrate the surrounding tissue and promote perivascular growth by angiogenesis or by vascular co-option (Kienast et al. 2010).

In addition to immunological responses, there is a need to continue developing technologies that deepen the ability to probe further into cell-cell communication in a way that surveys interactions at the molecular level.

7.4 Cell Fate

To identify the original cell population in vivo, reference points which allow identification of these cells overtime are needed (Bins et al. 2007). Due to the fast and ever-changing environment of a tissue formation/remodeling site, the use of reference points (e.g., fluorescent tags) that endure long enough to obtain an in-depth analysis of cell division and differentiation is restricted. Therefore, one major limitation of IVM is the ability to track the same cells over long periods of time (weeks) in different regions that the area originally analyzed. Some approaches have been emerging which may overcome this restraint. Two main methodologies have been development and applied which obtain remarkable results: photoswitchable proteins and color-engineered genetic constructs.

Photoactivatable fluorescent proteins (PAFPs) are molecules capable of changing their spectral properties in response to light excitation with a specific wavelength and intensity. Some PAFPs can undergo photoactivation where they convert from a low (dark) to a bright fluorescent state, whereas photoswitchable PAFPs change their emission wavelength depending on the value of the excitation wavelength (Lukyanov et al. 2005). To track cell fate and the development of an initial cell population, one approach is to use genetically engineered mice that express PAFPs. In a study performed by Kendrin et al., cells were modified to encode a photoswitchable protein termed Dendra2 (Kedrin et al. 2008). This protein possesses an

emission spectrum similar to GFP before undergoing a photoswitching process. After exposure to one-photon excitation wavelength (~405 nm), it undergoes an irreversible red shift (>150 nm) in both emission and excitation wavelength (Gurskaya et al. 2006). After photoswitching, the red fluorescence emission of Dendra2 increases, and after 5 days of the procedure, the red fluorescence intensity is still observable. Using this approach, heterogeneity of tumor microenvironments was observed. These differences were promoted by the presence of blood vessels within the same tumor which results in varying supplies of nutrients affecting the rate of cell invasion and tumor aggressiveness (Kedrin et al. 2008). Although this has been shown previously, the quantitative analysis of this variability was not compatible with previous techniques (Wyckoff et al. 2007).

The use of color-engineered genetic construct was first introduced by Livet et al. (Fig. 6iv) (Livet et al. 2007). In this study, genetic constructs were introduced into cells that would label clonal populations with distinct colors. This technology denoted Brainbow was based on a recombination system that determines the expression of multiple copies of constructs which code for different fluorescent protein. The process creates a mosaic with up to 90 colors. In this work, Brainbow was used to label neurons and follow their interactions and connectivity over time in vivo. Another variation of these genetic constructs has also been developed (Weber et al. 2011). To follow cell fate in vivo is still elusive; nonetheless, IVM results with PAFPs or genetic cassettes may be able to complement another genetic-based study that use specific molecular identifiers to track cell fate.

8 Conclusions

When considering how recent innovations in the microscopic fields have enabled invasive and noninvasive cell tracking, several common themes become apparent. In the case of multiphoton microscopy, it can increase the depth of imaging in a tissue and allow one to excite endogenous fluorophores without invasive manipulation. SHG can quantify collagen alignment and density, giving an added biomechanical inference to biological dynamics. FLIM captures photons emitted by a sample after laser excitation, resulting in both intensity measurements and fluorescence lifetimes, which can map the microenvironment of light-emitting molecules and cellular metabolism. Last but not least, IVM opens the possibility of in vivo cell and tissue tracking. Here, systemic responses and cell-cell interactions can be evaluated and when coupled with the techniques previously described can benchmark in vivo with in vitro studies revealing compelling and significant outcomes. This renaissance provides the identification of single-cell features, cell-cell communication, and cellular responses to dynamic microenvironments and internal/external stimuli both in vitro and in vivo. This is crucial for a deeper understanding of molecular and cellular biology that is translatable to immunology, cancer research, metabolism, and tissue engineering fields. With this, new technologies and therapeutic developments may arise targeting specific pathways by stimulation or inhibition in a cellular or molecular context. Microscopy is a steadfast technique which has improved and

broadened due to several technological advances in the optics and physics fields. "Seeing is believing" is still one of the main arguments that can exert confirmation of a scientific hypothesis. Therefore, interdisciplinary development of imaging technologies will undoubtedly promote a wider comprehension of in vitro and in vivo cell biology with the possibility to modulate cell behavior using tissue engineering and regenerative medicine approaches.

References

Abraham T, Hogg J (2010) Extracellular matrix remodeling of lung alveolar walls in three dimensional space identified using second harmonic generation and multiphoton excitation fluorescence. J Struct Biol 171(2):189–196

Abtin A, Jain R, Mitchell AJ, Roediger B, Brzoska AJ, Tikoo S, Cheng Q, Ng LG, Cavanagh LL, von Andrian UH, Hickey MJ, Firth N, Weninger W (2014) Perivascular macrophages mediate neutrophil recruitment during bacterial skin infection. Nat Immunol 15(1):45–53

Alam SR, Wallrabe H, Svindrych Z, Chaudhary AK, Christopher KG, Chandra D, Periasamy A (2017) Investigation of mitochondrial metabolic response to doxorubicin in prostate cancer cells: an NADH, FAD and tryptophan FLIM assay. Sci Rep 7(1):10451

Aldridge J, Pye EK (1976) Cell density dependence of oscillatory metabolism. Nature 259 (5545):670–671

Alexander S, Koehl GE, Hirschberg M, Geissler EK, Friedl P (2008) Dynamic imaging of cancer growth and invasion: a modified skin-fold chamber model. Histochem Cell Biol 130(6):1147–1154

Alfonso-Garcia A, Smith TD, Datta R, Luu TU, Gratton E, Potma EO, Liu WF (2016) Label-free identification of macrophage phenotype by fluorescence lifetime imaging microscopy. J Biomed Opt 21(4):46005

Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F (2007) Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 317(5838):666–670

Baird GS, Zacharias DA, Tsien RY (1999) Circular permutation and receptor insertion within green fluorescent proteins. Proc Natl Acad Sci U S A 96(20):11241–11246

Bardell D (2004) The biologists' forum: the invention of the microscope. Bios 75(2):78–84

Bataller R, Brenner DA (2005) Liver fibrosis. J Clin Invest 115(2):209–218

Bauer DE, Harris MH, Plas DR, Lum JJ, Hammerman PS, Rathmell JC, Riley JL, Thompson CB (2004) Cytokine stimulation of aerobic glycolysis in hematopoietic cells exceeds proliferative demand. FASEB J 18(11):1303–1305

Bedossa P, Poynard T (1996) An algorithm for the grading of activity in chronic hepatitis C. The METAVIR cooperative study group. Hepatology 24(2):289–293

Berg J, Hung YP, Yellen G (2009) A genetically encoded fluorescent reporter of ATP: ADP ratio. Nat Methods 6(2):161–166

Bins AD, van Rheenen J, Jalink K, Halstead JR, Divecha N, Spencer DM, Haanen JB, Schumacher TN (2007) Intravital imaging of fluorescent markers and FRET probes by DNA tattooing. BMC Biotechnol 7:2

Blacker TS, Duchen MR (2016) Investigating mitochondrial redox state using NADH and NADPH autofluorescence. Free Radic Biol Med 100:53–65

Blacker TS, Mann ZF, Gale JE, Ziegler M, Bain AJ, Szabadkai G, Duchen MR (2014) Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. Nat Commun 5:3936

Boddupalli A, Bratlie KM (2015) Multimodal imaging of harmonophores and application of high content imaging for early cancer detection. Mater Discov 1:10–20

Boddupalli A, Bratlie KM (2019) Second harmonic generation microscopy of collagen organization in tunable, environmentally responsive alginate hydrogels. Biomater Sci 7(3):1188–1199

Boens N, Qin W, Basaric N, Hofkens J, Ameloot M, Pouget J, Lefevre JP, Valeur B, Gratton E, vande Ven M, Silva ND Jr, Engelborghs Y, Willaert K, Sillen A, Rumbles G, Phillips D, Visser AJ, van Hoek A, Lakowicz JR, Malak H, Gryczynski I, Szabo AG, Krajcarski DT, Tamai N, Miura A (2007) Fluorescence lifetime standards for time and frequency domain fluorescence spectroscopy. Anal Chem 79(5):2137–2149

Botchway SW, Parker AW, Bisby RH, Crisostomo AG (2008) Real-time cellular uptake of serotonin using fluorescence lifetime imaging with two-photon excitation. Microsc Res Tech 71(4):267–273

Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. Biochem J 435 (2):297–312

Breart B, Lemaitre F, Celli S, Bousso P (2008) Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. J Clin Invest 118(4):1390–1397

Brown E, McKee T, diTomaso E, Pluen A, Seed B, Boucher Y, Jain RK (2003) Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. Nat Med 9 (6):796–800

Brown CM, Rivera DR, Pavlova I, Ouzounov DG, Williams WO, Mohanan S, Webb WW, Xu C (2012) In vivo imaging of unstained tissues using a compact and flexible multiphoton micro-endoscope. J Biomed Opt 17(4):040505

Bullen A (2008) Microscopic imaging techniques for drug discovery. Nat Rev Drug Discov 7 (1):54–67

Buranachai C, Kamiyama D, Chiba A, Williams BD, Clegg RM (2008) Rapid frequency-domain FLIM spinning disk confocal microscope: lifetime resolution, image improvement and wavelet analysis. J Fluoresc 18(5):929–942

Buurman EP, Sanders R, Draaijer A, Gerritsen HC, van Veen JJF, Houpt PM, Levine YK (1992) Fluorescence lifetime imaging using a confocal laser scanning microscope. Scanning 14 (3):155–159

Caetano-Lopes J, Nery AM, Henriques R, Canhão H, Duarte J, Amaral PM, Vale M, Moura RA, Pereira PA, Weinmann P, Abdulghani S, Souto-Carneiro M, Rego P, Monteiro J, Sakagushi S, Queiroz MV, Konttinen YT, Graça L, Vaz MF, Fonseca JE (2009) Chronic arthritis directly induces quantitative and qualitative bone disturbances leading to compromised biomechanical properties. Clin Exp Rheumatol 27(3):475–482

Campagnola P (2011) Second harmonic generation imaging microscopy: applications to diseases diagnostics. Anal Chem 83(9):3224–3231

Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN (2006) Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature 440(7086):890–895

Cavanagh LL, Weninger W (2008) Dendritic cell behaviour in vivo: lessons learned from intravital two-photon microscopy. Immunol Cell Biol 86(5):428–438

Chen X, Nadiarynkh O, Plotnikov S, Campagnola PJ (2012) Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. Nat Protoc 7(4):654–669

Chunqiang L, Pitsillides C, Runnels JM, Côté D, Lin CP (2010) Multiphoton microscopy of live tissues with ultraviolet autofluorescence. IEEE J Sel Topics Quantum Electron 16(3):516–523

Daemen A, Peterson D, Sahu N, McCord R, Du X, Liu B, Kowanetz K, Hong R, Moffat J, Gao M, Boudreau A, Mroue R, Corson L, O'Brien T, Qing J, Sampath D, Merchant M, Yauch R, Manning G, Settleman J, Hatzivassiliou G, Evangelista M (2015) Metabolite profiling stratifies pancreatic ductal adenocarcinomas into subtypes with distinct sensitivities to metabolic inhibitors. Proc Natl Acad Sci U S A 112(32):E4410–E4417

Dang CV (2010) Glutaminolysis: supplying carbon or nitrogen or both for cancer cells? Cell Cycle 9(19):3884–3886

DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7(1):11–20

Dempsey WP, Fraser SE, Pantazis P (2012) SHG nanoprobes: advancing harmonic imaging in biology. BioEssays 34(5):351–360

Diffey BL (1991) Solar ultraviolet radiation effects on biological systems. Phys Med Biol 36 (3):299–328

Digman MA, Caiolfa VR, Zamai M, Gratton E (2008) The phasor approach to fluorescence lifetime imaging analysis. Biophys J 94(2):L14–L16

Dimitrow E, Riemann I, Ehlers A, Koehler MJ, Norgauer J, Elsner P, Konig K, Kaatz M (2009) Spectral fluorescence lifetime detection and selective melanin imaging by multiphoton laser tomography for melanoma diagnosis. Exp Dermatol 18(6):509–515

Dmitriev RI (2017) Multi-parametric live cell microscopy of 3D tissue models. Springer, Cham

Dmitriev RI, Papkovsky DB (2015) Intracellular probes for imaging oxygen concentration: how good are they? Methods Appl Fluoresc 3(3):034001

Dmitriev RI, Papkovsky DB (2018) Quenched-phosphorescence detection of molecular oxygen: applications in life sciences. Royal Society of Chemistry, Cambridge

Dmitriev RI, Zhdanov AV, Nolan YM, Papkovsky DB (2013) Imaging of neurosphere oxygenation with phosphorescent probes. Biomaterials 34(37):9307–9317

Dmitriev RI, Kondrashina AV, Koren K, Klimant I, Zhdanov AV, Pakan JM, McDermott KW, Papkovsky DB (2014) Small molecule phosphorescent probes for O 2 imaging in 3D tissue models. Biomater Sci 2(6):853–866

Dowling K, Dayel MJ, Lever MJ, French PM, Hares JD, Dymoke-Bradshaw AK (1998) Fluorescence lifetime imaging with picosecond resolution for biomedical applications. Opt Lett 23 (10):810–812

Drury JL, Mooney DJ (2003) Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials 24(24):4337–4351

Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN (2008) Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. Immunity 28(2):271–284

Egen JG, Rothfuchs AG, Feng CG, Horwitz MA, Sher A, Germain RN (2011) Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. Immunity 34(5):807–819

Ehlers A, Riemann I, Stark M, Konig K (2007) Multiphoton fluorescence lifetime imaging of human hair. Microsc Res Tech 70(2):154–161

Elangovan M, Wallrabe H, Chen Y, Day RN, Barroso M, Periasamy A (2003) Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy. Methods 29(1):58–73

Emiliani V, Sanvitto D, Tramier M, Piolot T, Petrasek Z, Kemnitz K, Durieux C, Coppey-Moisan M (2003) Low-intensity two-dimensional imaging of fluorescence lifetimes in living cells. Appl Phys Lett 83(12):2471–2473

Folbergrová J, Ljunggren B, Norberg K, Siesjö BK (1974) Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex. Brain Res 80(2):265–279

Folmes CD, Dzeja PP, Nelson TJ, Terzic A (2012) Metabolic plasticity in stem cell homeostasis and differentiation. Cell Stem Cell 11(5):596–606

Funane T, Hou SS, Zoltowska KM, van Veluw SJ, Berezovska O, Kumar ATN, Bacskai BJ (2018) Selective plane illumination microscopy (SPIM) with time-domain fluorescence lifetime imaging microscopy (FLIM) for volumetric measurement of cleared mouse brain samples. Rev Sci Instrum 89(5):053705

Gadella TW Jr, Arndt-Jovin DJ, Jovin TM (1994) Visualization of lipid-receptor interactions on single cells by time-resolved imaging fluorescence microscopy. J Fluoresc 4(4):295–298

Gailhouste L, Le Grand Y, Odin C, Guyader D, Turlin B, Ezan F, Desille Y, Guilbert T, Bessard A, Fremin C, Theret N, Baffet G (2010) Fibrillar collagen scoring by second harmonic microscopy: a new tool in the assessment of liver fibrosis. J Hepatol 52(3):398–406

Ghukasyan VV, Kao F-J (2009) Monitoring cellular metabolism with fluorescence lifetime of reduced nicotinamide adenine dinucleotide. J Phys Chem C 113(27):11532–11540

Giesen U, Langner F, Mielke C, Mosconi M, Dirks WG (2011) Online imaging of initial DNA damages at the PTB microbeam. Radiat Prot Dosim 143(2–4):349–352

Goldring MB, Goldring SR (1990) Skeletal tissue response to cytokines. Clin Orthop Relat Res 258:245–276
Göppert-Mayer M (1931) Über Elementarakte mit zwei Quantensprüngen. Ann Phys 401(3):273–294
Gratton E, Breusegem S, Sutin J, Ruan Q, Barry N (2003) Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods. J Biomed Opt 8(3):381–390
Gurcan MN, Boucheron LE, Can A, Madabhushi A, Rajpoot NM, Yener B (2009) Histopathological image analysis: a review. IEEE Rev Biomed Eng 2:147–171
Gurskaya NG, Verkhusha VV, Shcheglov AS, Staroverov DB, Chepurnykh TV, Fradkov AF, Lukyanov S, Lukyanov KA (2006) Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol 24(4):461–465
Haeger A, Krause M, Wolf K, Friedl P (2014) Cell jamming: collective invasion of mesenchymal tumor cells imposed by tissue confinement. Biochim Biophys Acta 1840(8):2386–2395
Hato T, Winfree S, Day R, Sandoval RM, Molitoris BA, Yoder MC, Wiggins RC, Zheng Y, Dunn KW, Dagher PC (2017) Two-photon intravital fluorescence lifetime imaging of the kidney reveals cell-type specific metabolic signatures. J Am Soc Nephrol 28(8):2420–2430
Helmchen F, Denk W (2005) Deep tissue two-photon microscopy. Nat Methods 2(12):932–940
Hickman HD, Takeda K, Skon CN, Murray FR, Hensley SE, Loomis J, Barber GN, Bennink JR, Yewdell JW (2008) Direct priming of antiviral CD8+ T cells in the peripheral interfollicular region of lymph nodes. Nat Immunol 9(2):155–165
Hooke R (1665) Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses. With Observations and Inquiries Thereupon. Great Britain, The Royal Society.
Huang S, Heikal AA, Webb WW (2002) Two-photon fluorescence spectroscopy and microscopy of NAD(P)H and flavoprotein. Biophys J 82(5):2811–2825
Hung YP, Albeck JG, Tantama M, Yellen G (2011) Imaging cytosolic NADH-NAD(+) redox state with a genetically encoded fluorescent biosensor. Cell Metab 14(4):545–554
Ishikawa-Ankerhold HC, Ankerhold R, Drummen GP (2012) Advanced fluorescence microscopy techniques–FRAP, FLIP, FLAP, FRET and FLIM. Molecules 17(4):4047–4132
Jain RK, Munn LL, Fukumura D (2002) Dissecting tumour pathophysiology using intravital microscopy. Nat Rev Cancer 2(4):266–276
Janssen A, Beerling E, Medema R, van Rheenen J (2013) Intravital FRET imaging of tumor cell viability and mitosis during chemotherapy. PLoS One 8(5):e64029
Jenkins J, Dmitriev RI, Morten K, McDermott KW, Papkovsky DB (2015) Oxygen-sensing scaffolds for 3-dimensional cell and tissue culture. Acta Biomater 16:126–135
Jenkins J, Borisov SM, Papkovsky DB, Dmitriev RI (2016) Sulforhodamine nanothermometer for multiparametric fluorescence lifetime imaging microscopy. Anal Chem 88(21):10566–10572
Jenne CN, Wong CH, Petri B, Kubes P (2011) The use of spinning-disk confocal microscopy for the intravital analysis of platelet dynamics in response to systemic and local inflammation. PLoS One 6(9):e25109
Jyothikumar V, Sun Y, Periasamy A (2013) Investigation of tryptophan-NADH interactions in live human cells using three-photon fluorescence lifetime imaging and Forster resonance energy transfer microscopy. J Biomed Opt 18(6):060501
Kaiser W, Garrett CGB (1961) Two-photon excitation in CaF2:Eu2+. Phys Rev Lett 7(6):229–231
Kalinina S, Breymayer J, Schäfer P, Calzia E, Shcheslavskiy V, Becker W, Rück A (2016) Correlative NAD (P) H-FLIM and oxygen sensing-PLIM for metabolic mapping. J Biophotonics 9(8):800–811
Kautsky H, Hirsch A (1935) Nachweis geringster. Sauerstoffmengen durch Phosphoreszenztilgung 222(2):126–134
Kedrin D, Gligorijevic B, Wyckoff J, Verkhusha VV, Condeelis J, Segall JE, van Rheenen J (2008) Intravital imaging of metastatic behavior through a mammary imaging window. Nat Methods 5 (12):1019–1021

Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J, Winkler F (2010) Real-time imaging reveals the single steps of brain metastasis formation. Nat Med 16 (1):116–122

Kierdaszuk B, Gryczynski I, Modrak-Wojcik A, Bzowska A, Shugar D, Lakowicz JR (1995) Fluorescence of tyrosine and tryptophan in proteins using one- and two-photon excitation. Photochem Photobiol 61(4):319–324

Kondrashina AV, Dmitriev RI, Borisov SM, Klimant I, O'Brien I, Nolan YM, Zhdanov AV, Papkovsky DB (2012) A phosphorescent nanoparticle-based probe for sensing and imaging of (intra)cellular oxygen in multiple detection modalities. Adv Funct Mater 22(23):4931–4939

Konig K, Schenke-Layland K, Riemann I, Stock UA (2005) Multiphoton autofluorescence imaging of intratissue elastic fibers. Biomaterials 26(5):495–500

Lakowicz JR (1999) Principles of fluorescence spectroscopy. Kluwer academic, New York

Lakowicz JR, Maliwal BP (1983) Oxygen quenching and fluorescence depolarization of tyrosine residues in proteins. J Biol Chem 258(8):4794–4801

Lakowicz JR, Szmacinski H, Nowaczyk K, Johnson ML (1992) Fluorescence lifetime imaging of free and protein-bound NADH. Proc Natl Acad Sci U S A 89(4):1271–1275

Lammermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Soldner R, Hirsch K, Keller M, Forster R, Critchley DR, Fassler R, Sixt M (2008) Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature 453(7191):51–55

Lattouf R, Younes R, Lutomski D, Naaman N, Godeau G, Senni K, Changotade S (2014) Picrosirius red staining: a useful tool to appraise collagen networks in normal and pathological tissues. J Histochem Cytochem 62(10):751–758

Lawson MA, McDonald MM, Kovacic N, Hua Khoo W, Terry RL, Down J, Kaplan W, Paton-Hough J, Fellows C, Pettitt JA, Neil Dear T, Van Valckenborgh E, Baldock PA, Rogers MJ, Eaton CL, Vanderkerken K, Pettit AR, Quinn JM, Zannettino AC, Phan TG, Croucher PI (2015) Osteoclasts control reactivation of dormant myeloma cells by remodelling the endosteal niche. Nat Commun 6:8983

Leeuwenhoek Av (1682) Observationes microscopicae Antonii Lewenhoeck, circa particulas liquorum globosa et animalia. Leipzig, Acta Eruditorum.

Lilledahl MB, Pierce DM, Ricken T, Holzapfel GA, Davies Cde L (2011) Structural analysis of articular cartilage using multiphoton microscopy: input for biomechanical modeling. IEEE Trans Med Imaging 30(9):1635–1648

Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature 450(7166):56–62

Lleres D, James J, Swift S, Norman DG, Lamond AI (2009) Quantitative analysis of chromatin compaction in living cells using FLIM-FRET. J Cell Biol 187(4):481–496

Lukyanov KA, Chudakov DM, Lukyanov S, Verkhusha VV (2005) Innovation: photoactivatable fluorescent proteins. Nat Rev Mol Cell Biol 6(11):885–891

Lunt SY, Vander Heiden MG (2011) Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 27:441–464

Lutz V, Sattler M, Gallinat S, Wenck H, Poertner R, Fischer F (2012) Impact of collagen crosslinking on the second harmonic generation signal and the fluorescence lifetime of collagen autofluorescence. Skin Res Technol 18(2):168–179

Mantovani A, Dejana E (1989) Cytokines as communication signals between leukocytes and endothelial cells. Immunol Today 10(11):370–375

Mariani MM, Lampen P, Popp J, Wood BR, Deckert V (2009) Impact of fixation on in vitro cell culture lines monitored with Raman spectroscopy. Analyst 134(6):1154–1161

Marques PE, Oliveira AG, Chang L, Paula-Neto HA, Menezes GB (2015) Understanding liver immunology using intravital microscopy. J Hepatol 63(3):733–742

Masia R, McCarty WJ, Lahmann C, Luther J, Chung RT, Yarmush ML, Yellen G (2018) Live cell imaging of cytosolic NADH/NAD(+) ratio in hepatocytes and liver slices. Am J Physiol Gastrointest Liver Physiol 314(1):G97–G108

Masters BR, So PT, Gratton E (1997) Multiphoton excitation fluorescence microscopy and spectroscopy of in vivo human skin. Biophys J 72(6):2405–2412

McKeehan W (1982) Glycolysis, glutaminolysis and cell proliferation. Cell Biol Int Rep 6(7):635–650

Mempel TR, Scimone ML, Mora JR, von Andrian UH (2004) In vivo imaging of leukocyte trafficking in blood vessels and tissues. Curr Opin Immunol 16(4):406–417

Mempel TR, Pittet MJ, Khazaie K, Weninger W, Weissleder R, von Boehmer H, von Andrian UH (2006) Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. Immunity 25(1):129–141

Misgeld T, Kerschensteiner M (2006) In vivo imaging of the diseased nervous system. Nat Rev Neurosci 7(6):449–463

Mitchell CA, Poland SP, Seyforth J, Nedbal J, Gelot T, Huq T, Holst G, Knight RD, Ameer-Beg SM (2017) Functional in vivo imaging using fluorescence lifetime light-sheet microscopy. Opt Lett 42(7):1269–1272

Molitoris BA, Sandoval RM (2005) Intravital multiphoton microscopy of dynamic renal processes. Am J Physiol Renal Physiol 288(6):F1084–F1089

Monaghan MG, Kroll S, Brucker SY, Schenke-Layland K (2016) Enabling multiphoton and second harmonic generation imaging in paraffin-embedded and histologically stained sections. Tissue Eng Part C Methods 22(6):517–523

Mongeon R, Venkatachalam V, Yellen G (2016) Cytosolic NADH-NAD+ redox visualized in brain slices by two-photon fluorescence lifetime biosensor imaging. Antioxid Redox Signal 25 (10):553–563

Mookerjee SA, Gerencser AA, Nicholls DG, Brand MD (2017) Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. J Biol Chem 292(17):7189–7207

Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK (2000) Role of extracellular matrix assembly in interstitial transport in solid tumors. Cancer Res 60(9):2497–2503

Niesner R, Peker B, Schlusche P, Gericke KH (2004) Noniterative biexponential fluorescence lifetime imaging in the investigation of cellular metabolism by means of NAD(P)H autofluorescence. ChemPhysChem 5(8):1141–1149

Nobis M, Herrmann D, Warren SC, Kadir S, Leung W, Killen M, Magenau A, Stevenson D, Lucas MC, Reischmann N, Vennin C, Conway JRW, Boulghourjian A, Zaratzian A, Law AM, Gallego-Ortega D, Ormandy CJ, Walters SN, Grey ST, Bailey J, Chtanova T, Quinn JMW, Baldock PA, Croucher PI, Schwarz JP, Mrowinska A, Zhang L, Herzog H, Masedunskas A, Hardeman EC, Gunning PW, Del Monte-Nieto G, Harvey RP, Samuel MS, Pajic M, McGhee EJ, Johnsson AE, Sansom OJ, Welch HCE, Morton JP, Strathdee D, Anderson KI, Timpson P (2017) A RhoA-FRET biosensor mouse for intravital imaging in normal tissue homeostasis and disease contexts. Cell Rep 21(1):274–288

Nomoto T, Matsumoto Y, Miyata K, Oba M, Fukushima S, Nishiyama N, Yamasoba T, Kataoka K (2011) In situ quantitative monitoring of polyplexes and polyplex micelles in the blood circulation using intravital real-time confocal laser scanning microscopy. J Control Release 151(2):104–109

O'Donnell N, Dmitriev RI (2017) Three-dimensional tissue models and available probes for multiparametric live cell microscopy: a brief overview. In: Multi-parametric live cell microscopy of 3D tissue models. Springer, Cham, pp 49–67

O'Donnell N, Okkelman IA, Timashev P, Gromovykh TI, Papkovsky DB, Dmitriev RI (2018) Cellulose-based scaffolds for fluorescence lifetime imaging-assisted tissue engineering. Acta Biomater 80:85–96

O'Neill LA, Kishton RJ, Rathmell J (2016) A guide to immunometabolism for immunologists. Nat Rev Immunol 16(9):553–565

Ochoa LF, Kholodnykh A, Villarreal P, Tian B, Pal R, Freiberg AN, Brasier AR, Motamedi M, Vargas G (2018) Imaging of murine whole lung fibrosis by large scale 3D microscopy aided by tissue optical clearing. Sci Rep 8(1):13348

Okada T, Cyster JG (2007) CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. J Immunol 178(5):2973–2978

Okkelman IA, Dmitriev RI, Foley T, Papkovsky DB (2016) Use of fluorescence lifetime imaging microscopy (FLIM) as a timer of cell cycle S phase. PLoS One 11(12):e0167385

Okkelman IA, Foley T, Papkovsky DB, Dmitriev RI (2017) Live cell imaging of mouse intestinal organoids reveals heterogeneity in their oxygenation. Biomaterials 146:86–96

Okkelman IA, Neto N, Papkovsky DB, Monaghan MG, Dmitriev RI (2019a) A deeper understanding of intestinal organoid metabolism revealed by combining fluorescence lifetime imaging microscopy (FLIM) and extracellular flux analyses. Redox Biol 30:101420

Okkelman IA, Papkovsky DB, Dmitriev RI (2019b) Estimation of the mitochondrial membrane potential using fluorescence lifetime imaging microscopy. Cytometry A. https://doi.org/10.1002/cyto.a.23886

Orth JD, Kohler RH, Foijer F, Sorger PK, Weissleder R, Mitchison TJ (2011) Analysis of mitosis and antimitotic drug responses in tumors by in vivo microscopy and single-cell pharmacodynamics. Cancer Res 71(13):4608–4616

Ostrander JH, McMahon CM, Lem S, Millon SR, Brown JQ, Seewaldt VL, Ramanujam N (2010) Optical redox ratio differentiates breast cancer cell lines based on estrogen receptor status. Cancer Res 70(11):4759–4766

Pantazis P, Maloney J, Wu D, Fraser SE (2010) Second harmonic generating (SHG) nanoprobes for in vivo imaging. Proc Natl Acad Sci U S A 107(33):14535–14540

Panyi G, Bagdany M, Bodnar A, Vamosi G, Szentesi G, Jenei A, Matyus L, Varga S, Waldmann TA, Gaspar R, Damjanovich S (2003) Colocalization and nonrandom distribution of Kv1.3 potassium channels and CD3 molecules in the plasma membrane of human T lymphocytes. Proc Natl Acad Sci U S A 100(5):2592–2597

Papkovsky DB, Dmitriev RI (2013) Biological detection by optical oxygen sensing. Chem Soc Rev 42(22):8700–8732

Papkovsky DB, Dmitriev RI (2018) Imaging of oxygen and hypoxia in cell and tissue samples. Cell Mol Life Sci 75(16):2963–2980

Pena AM, Boulesteix T, Dartigalongue T, Schanne-Klein MC (2005) Chiroptical effects in the second harmonic signal of collagens I and IV. J Am Chem Soc 127(29):10314–10322

Phillips D, Drake RC, Oconnor DV, Christensen RL (1985) Time correlated single-photon counting (Tcspc) using laser excitation. Anal Instrum 14(3–4):267–292

Plotnikov SV, Millard AC, Campagnola PJ, Mohler WA (2006) Characterization of the myosin-based source for second-harmonic generation from muscle sarcomeres. Biophys J 90(2):693–703

Pollak N, Dölle C, Ziegler M (2007) The power to reduce: pyridine nucleotides – small molecules with a multitude of functions. Biochem J 402(2):205–218

Prockop DJ, Fertala A (1998) The collagen fibril: the almost crystalline structure. J Struct Biol 122 (1–2):111–118

Progatzky F, Dallman MJ, Lo Celso C (2013) From seeing to believing: labelling strategies for in vivo cell-tracking experiments. Interface Focus 3(3):20130001

Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ (2006) Collagen reorganization at the tumor-stromal interface facilitates local invasion. BMC Med 4(1):38–54

Provenzano PP, Eliceiri KW, Keely PJ (2009) Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. Clin Exp Metastasis 26(4):357–370

Pugh TD, Conklin MW, Evans TD, Polewski MA, Barbian HJ, Pass R, Anderson BD, Colman RJ, Eliceiri KW, Keely PJ, Weindruch R, Beasley TM, Anderson RM (2013) A shift in energy metabolism anticipates the onset of sarcopenia in rhesus monkeys. Aging Cell 12 (4):672–681

Puxkandl R, Zizak I, Paris O, Keckes J, Tesch W, Bernstorff S, Purslow P, Fratzl P (2002) Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. Philos Trans R Soc Lond Ser B Biol Sci 357(1418):191–197

Quinn KP, Bellas E, Fourligas N, Lee K, Kaplan DL, Georgakoudi I (2012) Characterization of metabolic changes associated with the functional development of 3D engineered tissues by non-invasive, dynamic measurement of individual cell redox ratios. Biomaterials 33(21):5341–5348

Radbruch H, Bremer D, Mothes R, Gunther R, Rinnenthal JL, Pohlan J, Ulbricht C, Hauser AE, Niesner R (2015) Intravital FRET: probing cellular and tissue function in vivo. Int J Mol Sci 16 (5):11713–11727

Ranjit S, Dobrinskikh E, Montford J, Dvornikov A, Lehman A, Orlicky DJ, Nemenoff R, Gratton E, Levi M, Furgeson S (2016) Label-free fluorescence lifetime and second harmonic generation imaging microscopy improves quantification of experimental renal fibrosis. Kidney Int 90 (5):1123–1128

Rinnenthal JL, Bornchen C, Radbruch H, Andresen V, Mossakowski A, Siffrin V, Seelemann T, Spiecker H, Moll I, Herz J, Hauser AE, Zipp F, Behne MJ, Niesner R (2013) Parallelized TCSPC for dynamic intravital fluorescence lifetime imaging: quantifying neuronal dysfunction in neuroinflammation. PLoS One 8(4):e60100

Robert Lanza RLAJV (2014) Principles of tissue engineering. Elsevier, London

Roussakis E, Li Z, Nichols AJ, Evans CLJACIE (2015) Oxygen-sensing methods in biomedicine from the macroscale to the microscale. Angew Chem Int Ed 54(29):8340–8362

Roussakis E, Ortines RV, Pinsker BL, Mooers CT, Evans CL, Miller LS, Calderón-Colón XJB (2019) Theranostic biocomposite scaffold membrane. Biomaterials 212:17–27

Roussos ET, Condeelis JS, Patsialou A (2011) Chemotaxis in cancer. Nat Rev Cancer 11(8):573–587

Sakadžić S, Roussakis E, Yaseen MA, Mandeville ET, Srinivasan VJ, Arai K, Ruvinskaya S, Devor A, Lo EH, Vinogradov SA (2010) Two-photon high-resolution measurement of partial pressure of oxygen in cerebral vasculature and tissue. Nat Methods 7(9):755

Saraste M (1999) Oxidative phosphorylation at the fin de siecle. Science 283(5407):1488–1493

Scaduto RC, Grotyohann LW (1999) Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 76(1):469–477

Schaefer PM, Kalinina S, Rueck A, von Arnim CAF, von Einem B (2019) NADH autofluorescence-a marker on its way to boost bioenergetic research. Cytometry A 95(1):34–46

Schenke-Layland K, Madershahian N, Riemann I, Starcher B, Halbhuber KJ, Konig K, Stock UA (2006) Impact of cryopreservation on extracellular matrix structures of heart valve leaflets. Ann Thorac Surg 81(3):918–926

Schilling K, El Khatib M, Plunkett S, Xue J, Xia Y, Vinogradov SA, Brown E, Zhang X (2019) Electrospun fiber mesh for high-resolution measurements of oxygen tension in cranial bone defect repair. ACS Appl Mater Interfaces 11:33548–33558

Schneider PC, Clegg RM (1997) Rapid acquisition, analysis, and display of fluorescence lifetime-resolved images for real-time applications. Rev Sci Instrum 68(11):4107–4119

Sekar RB, Periasamy A (2003) Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. J Cell Biol 160(5):629–633

Sereysky JB, Andarawis-Puri N, Jepsen KJ, Flatow EL (2012) Structural and mechanical effects of in vivo fatigue damage induction on murine tendon. J Orthop Res 30(6):965–972

Shear JB, Xu C, Webb WW (1997) Multiphoton-excited visible emission by serotonin solutions. Photochem Photobiol 65(6):931–936

Shen YR (1984) The principles of nonlinear optics. Wiley, New York

Shestov AA, Liu X, Ser Z, Cluntun AA, Hung YP, Huang L, Kim D, Le A, Yellen G, Albeck JG, Locasale JW (2014) Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. eLife 3:e03342. https://doi.org/10.7554/eLife.03342

Skala MC, Riching KM, Gendron-Fitzpatrick A, Eickhoff J, Eliceiri KW, White JG, Ramanujam N (2007) In vivo multiphoton microscopy of NADH and FAD redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia. Proc Natl Acad Sci U S A 104(49):19494–19499

Sommer G, Schriefl AJ, Andra M, Sacherer M, Viertler C, Wolinski H, Holzapfel GA (2015) Biomechanical properties and microstructure of human ventricular myocardium. Acta Biomater 24:172–192

Spencer SL, Gaudet S, Albeck JG, Burke JM, Sorger PK (2009) Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. Nature 459(7245):428–432

Squire A, Bastiaens PI (1999) Three dimensional image restoration in fluorescence lifetime imaging microscopy. J Microsc 193(Pt 1):36–49

Stanciu SG, Xu S, Peng Q, Yan J, Stanciu GA, Welsch RE, So PT, Csucs G, Yu H (2014) Experimenting liver fibrosis diagnostic by two photon excitation microscopy and bag-of-features image classification. Sci Rep 4:4636

Stoller P, Celliers PM, Reiser KM, Rubenchik AM (2003) Quantitative second-harmonic generation microscopy in collagen. Appl Opt 42(25):5209–5219

Stoscheck CM (1990) Quantitation of protein. Methods Enzymol 182:50–68

Strupler M, Pena AM, Hernest M, Tharaux PL, Martin JL, Beaurepaire E, Schanne-Klein MC (2007) Second harmonic imaging and scoring of collagen in fibrotic tissues. Opt Express 15 (7):4054–4065

Sumen C, Mempel TR, Mazo IB, von Andrian UH (2004) Intravital microscopy: visualizing immunity in context. Immunity 21(3):315–329

Sytsma J, Vroom JM, de Grauw CJ, Gerritsen HC (2008) Time-gated fluorescence lifetime imaging and microvolume spectroscopy using two-photon excitation. J Microsc 191(1):39–51

Tang Q, Adams JY, Tooley AJ, Bi M, Fife BT, Serra P, Santamaria P, Locksley RM, Krummel MF, Bluestone JA (2006) Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol 7(1):83–92

Tantama M, Hung YP, Yellen G (2011) Imaging intracellular pH in live cells with a genetically encoded red fluorescent protein sensor. J Am Chem Soc 133(26):10034–10037

Thai M, Graham NA, Braas D, Nehil M, Komisopoulou E, Kurdistani SK, McCormick F, Graeber TG, Christofk HR (2014) Adenovirus E4ORF1-induced MYC activation promotes host cell anabolic glucose metabolism and virus replication. Cell Metab 19(4):694–701

Thestrup T, Litzlbauer J, Bartholomaus I, Mues M, Russo L, Dana H, Kovalchuk Y, Liang Y, Kalamakis G, Laukat Y, Becker S, Witte G, Geiger A, Allen T, Rome LC, Chen TW, Kim DS, Garaschuk O, Griesinger C, Griesbeck O (2014) Optimized ratiometric calcium sensors for functional in vivo imaging of neurons and T lymphocytes. Nat Methods 11(2):175–182

Thorling CA, Liu X, Burczynski FJ, Fletcher LM, Roberts MS, Sanchez WY (2013) Intravital multiphoton microscopy can model uptake and excretion of fluorescein in hepatic ischemia-reperfusion injury. J Biomed Opt 18(10):101306

Timpson P, McGhee EJ, Anderson KI (2011) Imaging molecular dynamics in vivo-from cell biology to animal models. J Cell Sci 124(Pt 17):2877–2890

Valentini G, D'Andrea C, Comelli D, Pifferi A, Taroni P, Torricelli A, Cubeddu R, Battaglia C, Consolandi C, Salani G, Rossi-Bernardi L, De Bellis G (2000) Time-resolved DNA-microarray reading by an intensified CCD for ultimate sensitivity. Opt Lett 25(22):1648–1650

van Munster E.B., Gadella T.W.J. (2005) Fluorescence Lifetime Imaging Microscopy (FLIM). In: Rietdorf J. (eds) Microscopy Techniques. Advances in Biochemical Engineering, vol 95. Springer, Berlin, Heidelberg. https://doi.org/10.1007/b102213

Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324(5930):1029–1033

Venkatasubramanian RT, Wolkers WF, Shenoi MM, Barocas VH, Lafontaine D, Soule CL, Iaizzo PA, Bischof JC (2010) Freeze-thaw induced biomechanical changes in arteries: role of collagen matrix and smooth muscle cells. Ann Biomed Eng 38(3):694–706

Vielreicher M, Schurmann S, Detsch R, Schmidt MA, Buttgereit A, Boccaccini A, Friedrich O (2013) Taking a deep look: modern microscopy technologies to optimize the design and functionality of biocompatible scaffolds for tissue engineering in regenerative medicine. J R Soc Interface 10(86):20130263

Wallrabe H, Periasamy A (2005) Imaging protein molecules using FRET and FLIM microscopy. Curr Opin Biotechnol 16(1):19–27

Wallrabe H, Elangovan M, Burchard A, Periasamy A, Barroso M (2003) Confocal FRET microscopy to measure clustering of ligand-receptor complexes in endocytic membranes. Biophys J 85 (1):559–571

Wallrabe H, Svindrych Z, Alam SR, Siller KH, Wang T, Kashatus D, Hu S, Periasamy A (2018) Segmented cell analyses to measure redox states of autofluorescent NAD(P)H, FAD & Trp in cancer cells by FLIM. Sci Rep 8(1):79

Walsh A, Cook RS, Rexer B, Arteaga CL, Skala MC (2012) Optical imaging of metabolism in HER2 overexpressing breast cancer cells. Biomed Opt Express 3(1):75–85
Walsh AJ, Cook RS, Manning HC, Hicks DJ, Lafontant A, Arteaga CL, Skala MC (2013) Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. Cancer Res 73(20):6164–6174
Weber K, Thomaschewski M, Warlich M, Volz T, Cornils K, Niebuhr B, Tager M, Lutgehetmann M, Pollok JM, Stocking C, Dandri M, Benten D, Fehse B (2011) RGB marking facilitates multicolor clonal cell tracking. Nat Med 17(4):504–509
Wilt BA, Burns LD, Wei Ho ET, Ghosh KK, Mukamel EA, Schnitzer MJ (2009) Advances in light microscopy for neuroscience. Annu Rev Neurosci 32:435–506
Winkler M, Chai D, Kriling S, Nien CJ, Brown DJ, Jester B, Juhasz T, Jester JV (2011) Nonlinear optical macroscopic assessment of 3-D corneal collagen organization and axial biomechanics. Invest Ophthalmol Vis Sci 52(12):8818–8827
Wolfbeis OSJB (2015) Luminescent sensing and imaging of oxygen: fierce competition to the Clark electrode. BioEssays 37(8):921–928
Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J (2004) A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 64(19):7022–7029
Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, Segall JE, Pollard JW, Condeelis J (2007) Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res 67(6):2649–2656
Xue R, Behera P, Xu J, Viapiano MS, Lannutti JJ (2014) Polydimethylsiloxane core–polycaprolactone shell nanofibers as biocompatible, real-time oxygen sensors. Sensors Actuators B Chem 192:697–707
Xue R, Ge C, Richardson K, Palmer A, Viapiano M, Lannutti JJ (2015) Microscale sensing of oxygen via encapsulated porphyrin nanofibers: effect of indicator and polymer “core” permeability. ACS Appl Mater Interfaces 7(16):8606–8614
Yaseen MA, Sakadzic S, Wu W, Becker W, Kasischke KA, Boas DA (2013) In vivo imaging of cerebral energy metabolism with two-photon fluorescence lifetime microscopy of NADH. Biomed Opt Express 4(2):307–321
Yazgan G, Dmitriev RI, Tyagi V, Jenkins J, Rotaru G-M, Rottmar M, Rossi RM, Toncelli C, Papkovsky DB, Maniura-Weber K (2017) Steering surface topographies of electrospun fibers: understanding the mechanisms. Sci Rep 7:158. https://doi.org/10.1038/s41598-017-00181-0
Ying W (2008) NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxid Redox Signal 10(2):179–206
Yoshihara T, Hirakawa Y, Hosaka M, Nangaku M, Tobita S (2017) Oxygen imaging of living cells and tissues using luminescent molecular probes. J Photochem Photobiol C: Photochem Rev 30:71–95
Zaritsky A, Natan S, Horev J, Hecht I, Wolf L, Ben-Jacob E, Tsarfaty I (2011) Cell motility dynamics: a novel segmentation algorithm to quantify multi-cellular bright field microscopy images. PLoS One 6(11):e27593
Zipfel WR, Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW (2003a) Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. Proc Natl Acad Sci U S A 100(12):7075–7080
Zipfel WR, Williams RM, Webb WW (2003b) Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol 21(11):1369–1377

Part IV

Mode of Cell Action

Mesenchymal Stromal Cell Secretome for Tissue Repair

Massimiliano Gnecchi, Maria Chiara Ciuffreda, and Manuela Mura

Contents

Abstract

Mesenchymal stromal cells (MSCs) are adult multipotent stem cells that can be easily harvested from a variety of human tissues and have the capacity to differentiate in multiple cell lineages. From a therapeutic perspective, MSC plasticity and low immunogenicity render these cells suitable for cell therapy and tissue engineering. Tissue regeneration from transplanted MSCs was originally proposed as the principal mechanism underlying their therapeutic action. However, recent studies have demonstrated that, instead of differentiating into target tissue types, stem cells exert their therapeutic effects via the secretion of

M. Gnecchi (✉) · M. Mura
Coronary Care Unit, Laboratory of Experimental Cardiology for Cell and Molecular Therapy, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

Department of Molecular Medicine, Unit of Cardiology, University of Pavia, Pavia, Italy
e-mail: m.gnecchi@unipv.it; m.mura@smatteo.pv.it

M. C. Ciuffreda
Coronary Care Unit, Laboratory of Experimental Cardiology for Cell and Molecular Therapy, IRCCS Policlinico San Matteo Foundation, Pavia, Italy
e-mail: mariachiara.ciuffreda@unipv.it

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_43

bioactive factors with anti-apoptotic, anti-scarring, pro-vasculogenic and immunomodulatory effects. This concept is supported by preclinical studies demonstrating equal or even improved organ function upon infusion of MSC secretome compared with MSC transplantation. In this chapter we will describe key MSC-secreted factors and their functional role on cardiovascular, renal, liver, and neurodegenerative disease models. A better characterization and understanding of MSC secretome will also be discussed.

1 Introduction

The study of stem cells in regenerative medicine is a growing field of basic and clinical research, generating a broad interest and debate in the scientific and the public communities. The ability to mobilize and activate endogenous stem/progenitor cells in diseased organs or to introduce exogenous stem cells for tissue regeneration/repair may positively impact many diseases.

Starting from the late 1990s, the regenerative capacity of a variety of multipotent adult stem cells (ASCs) harvested from different sources has been experimentally tested both in vitro and in vivo. Among ASCs, the discovery of a pool of multipotent adult progenitor cells, named mesenchymal stromal cells (MSCs), has generated remarkable interest.

MSCs are undifferentiated adult cells derived from mesodermal embryonic layer that can differentiate into a broad range of different mesenchymal tissues, including cartilage, bone, muscle, stroma, fat, tendon, and other connective tissues (Uccelli et al. 2008; Ciuffreda et al. 2016). These cells have been originally isolated from bone marrow (BM) where they regulate self-renewal, maturation, and recruitment of hematopoietic stem cells to the vascular compartment (Mareschi et al. 2001). In the 1960s Ernest A. McCulloch and James E. Till first revealed the clonal nature of marrow stromal cells (Mcculloch and Till 1960; Till and McCulloch 1961), and in the 1970s Friedenstein and colleagues reported their clonogenic potentiality in vitro (Friedenstein et al. 1968, 1970, 1974). In 1999, Pittenger et al. demonstrated that these cells are multipotent stem cells with potential to differentiate into other cells from mesenchymal tissues (Pittenger et al. 1999). Today, it is known that MSCs can be isolated not only from the bone marrow but also from cord blood, placenta, skeletal muscle, dental pulp, amniotic membrane, and adipose tissue (Erices et al. 2000; Gronthos et al. 2001; In ‘t et al. 2003; Ding et al. 2011). Circulating MSCs have also been described, but the results are debated and not always reproducible (Roufosseet al. 2004). It is important to note that the origin of MSCs may determine their fate and functional characteristics (Keyser et al. 2007). Panepucci et al. demonstrated that MSCs from bone marrow expressed more genes related to osteogenesis, whereas MSCs from umbilical cord expressed genes related to matrix remodeling and angiogenesis (Panepucci et al. 2004). However, this is not a dogma and, regardless of where they are isolated from, MSCs can be induced to differentiate into a broad range of different cells depending on culture conditions (Jaiswal et al. 1997; Johnstone et al. 1998; Wakitani et al. 1995). For instance, in vitro it is possible

to differentiate human MSCs into cardiac-like cells (CMC) using chemical compounds (Makino et al. 1999; Martin-Rendon et al. 2008) or by overexpressing specific miRNAs (Pisano et al. 2015). However, it is important to note that in vitro differentiation and marker expression are not equivalent to functional in vivo capacity as carefully reviewed by Bianco and colleagues (Bianco et al. 2008, 2013; Robey 2017).

Accordingly, tissue regeneration from transdifferentiation of transplanted MSCs was originally proposed as the principal mechanism underlying their therapeutic effects (Tomita et al. 1999; Kinnaird et al. 2004; Morigi et al. 2004, 2008; Pittenger and Martin 2004; Uccelli et al. 2008; Morigi and De Coppi 2014). However, many investigators reported that there is no evidence that MSCs are able to efficiently differentiate in vivo. For example, researchers have failed to detect permanent engraftment of transplanted BM-MSCs (Muller-Ehmsen et al. 2006) and MSC transdifferentiation into contractile cardiomyocytes (Toma et al. 2002). Moreover, exogenously administered MSCs show poor survival and do not persist at the site of myocardial infarction (Iso et al. 2007; Terrovitis et al. 2010), probably as a consequence of the harsh ischemic microenvironment, characterized by oxidative stress, inflammation, cytotoxic cytokines, and, in some instances, the absence of extracellular matrix (ECM) for MSC attachment (Rodrigues et al. 2010; Song et al. 2010). Furthermore, BM-MSC-based therapy for the treatment of central nervous system (CNS) diseases showed that differentiation into neuronal cells is extremely low and it is not the sole approach for transplanted stem cells to foster tissue repair in vivo (Chen et al. 2001; Pluchino and Cossetti 2013). In addition, it has been shown that few cells survive after 2 weeks from transplant (Mora-Lee et al. 2012) and long-term cell engraftment results are negligible (Chen et al. 2001).

The therapeutic action of BM-MSCs was also tested in a model of renal damage showing that the cells increased renal function but not through differentiation into tubular cells (Duffield et al. 2005; Lange et al. 2005; Togel et al. 2005, 2007). Moreover, despite the renal functional restoration, it was demonstrated that only a reduced percentage of injected BM-MSC was engrafted in the renal tissue and that these few cells were preferentially localized at the peritubular level and, less frequently, in the tubular epithelium (Morigi et al. 2004, 2008; Imberti et al. 2007).

Recently, there has been much interest in the development of MSC-based therapies for wound repair. Also in this case, there is an open debate on the ability of these cells to differentiate. Many authors showed cell differentiation into epidermal keratinocytes, pericytes, and sebocytes (Li et al. 2006; Wu et al. 2007; Sasaki et al. 2008); other investigators reported that there is no evidence that MSCs transdifferentiate into skin cells in a wound healing model (Javazon et al. 2007).

All these reports question the plasticity of transplanted MSCs. Regardless of whether stem cells can transdifferentiate, it has been shown that in many cases the amount of newly generated tissue is too limited to justify functional improvements. Therefore, recent studies have demonstrated that, instead of differentiating into target tissue types, stem cells exert a therapeutic effect via the secretion of bioactive factors that have anti-apoptotic, anti-scarring, and pro-vasculogenic effects, as well as immunomodulatory properties (Kinnaird et al. 2004; Gnecchi et al. 2005, 2006;

Togel et al. 2005; Bi et al. 2007; Morigi et al. 2008; Jung et al. 2009; Wakabayashi et al. 2010; Du et al. 2012; Fang et al. 2012; Morigi and De Coppi 2014). The new emerging view, which is to foster the repair of damaged tissue by harnessing paracrine factors instead of using whole cells, introduces a different dimension to the therapeutic application of MSCs in regenerative medicine. This has directed the scientific community to the challenging investigation of the molecules that make up the stem cell secretome. Regardless of whether these molecules are soluble or delivered by extracellular vesicles, the secretome are responsible for most of the beneficial effects observed after stem cell transplantation.

2 MSC Secretome in Regenerative Medicine

There is a growing interest in understanding how stem cells work via paracrine actions. The paracrine signaling involved by cells is composed of secreted molecules mediating cytoprotective, vasculogenic, anti-fibrotic, and anti-inflammatory effects. We now summarize the major findings regarding MSC-mediated paracrine actions in different pathological conditions.

2.1 Myocardial Injury

Coronary artery disease and heart failure are leading causes of mortality and morbidity worldwide. Accordingly, many investigators have focused their efforts in this area of research, and it has been proposed that paracrine mechanisms mediated by factors released by MSC play an essential role in the reparative processes observed after stem cell injection into infarcted hearts (Gnecchi et al. 2008). Gnecchi and colleagues were the first to demonstrate that the stem cell secretome obtained from MSCs overexpressing the gene Akt-1 (Akt-MSCs) exerted a cytoprotective effect when injected in a rat experimental model of permanent coronary occlusion. The data obtained showed a preservation of cardiac function and a significant limitation in infarct size and apoptotic index compared with controls, confirming that cytoprotection was the main mechanism of stem cell action (Gnecchi et al. 2006). Cytoprotective effects exerted by MSCs derived from different sources were confirmed by other investigators (Takahashi et al. 2006; Uemura et al. 2006; Xu et al. 2007; Danieli et al. 2015) and replicated in clinically relevant models (Lim et al. 2006). Another important biological process positively influenced by stem cells in a paracrine fashion is neovascularization. Despite evidence that BM-MSCs incorporate into vascular structures, only a small number of vessels contain donor cells. However, many studies testing MSC transplantation in experimental MI models reported an increase in capillary density in treated animals (Tomita et al. 2002; Nagaya et al. 2004; Jiang et al. 2006). Moreover, when MSC-conditioned medium (MSC-CM) was injected in a cardiac model of ischemia/reperfusion injury (I/R), it resulted in a significant increase of vascular density compared with controls

(Danieli et al. 2015), suggesting that MSCs represent a source of paracrine pro-angiogenic and pro-arteriogenic factors.

Besides cytoprotection and neovascularization, paracrine factors released by the transplanted stem cell may alter the extracellular matrix, resulting in a better postinfarction remodeling and strengthening of the infarct scar. For instancce, MSC injection into ischemic rat hearts decreased fibrosis, apoptosis, and left ventricular (LV) dilatation, whereas it increased myocardial thickness (Berry et al. 2006). Consistently, it has been shown that MSCs express molecules involved in the biogenesis of extracellular matrix such as collagens, matrix metalloproteinases (MMP), serine proteases, and serine protease inhibitors (Ohnishi et al. 2007b). It has been reported that injection of MSCs into infarcted hearts attenuated the increased cardiac expression of collagen types I and III, tissue inhibitor of metalloproteinase (TIMP)-1, transforming growth factor (TGF)-β, and cardiac fibroblast proliferation and that MSC-CM attenuates cardiac fibroblast proliferation and collagen synthesis in vitro (Xu et al. 2005; Ohnishi et al. 2007a). Nagaya and colleagues demonstrated that transplantation of BM-MSCs in an experimental model of dilated cardiomyopathy significantly increased capillary density and, most importantly, decreased the collagen deposition in the myocardium, resulting in decreased LV end-diastolic pressure and increased LV maximum dP/dt (Nagaya et al. 2005). Recently, we have shown that both the cytoprotective and the pro-angiogenic actions can be empowered by administering MSCs together with a synthetic extracellular matrix mimic, a hydrogel containing heparin (H-HG), able to improve cell survival but also to bind growth factors produced by the MSCs, such as VEGF and FGF, and release them over time (Ciuffreda et al. 2018).

It has been originally hypothesized that, when injected into the injured myocardium, MSCs proliferate and transdifferentiate into cardiomyocytes or fuse with native cardiomyocytes, thereby regenerating the lost myocardium (Dimmeler et al. 2005; Gnecchi et al. 2012, 2016). However, an intriguing new hypothesis has recently emerged indicating that transplanted MSCs, rather than by direct regeneration, might favour cardiac repopulation by activating resident cardiac progenitor cells (CPC) and/or by stimulating CMC replication via paracrine action (Hatzistergos et al. 2010). In support of this hypothesis, it has been shown that, under hypoxic conditions, MSCs are able to secrete hepatocyte growth factor (HGF) and insulin-like growth factor (IGF-1) (Gnecchi et al. 2006), two important molecules able to induce CPC migration, proliferation, and differentiation when administered into the myocardium (Linke et al. 2005). Hatzistergos et al. showed direct evidence that BM-MSCs may stimulate proliferation and differentiation of endogenous CPC (Hatzistergos et al. 2010). The authors observed that when MSCs were injected in a myocardial I/R injury model, endogenous CMC were regenerated by endogenous c-kit^{+} CPC and by replicating native CMC.

Interestingly, there is evidence suggesting that the administration of MSCs positively may influence also cardiac contractility. In this regard, it is known that, in addition to its growth-promoting and anti-apoptotic actions, IGF-1 can increase cardiomyocyte contractility in vitro (Freestone et al. 1996) and improve myocardial function both in normal and infarcted adult rat hearts (Duerr et al. 1995). Feygin et al.

showed that MSC transplantation significantly improved contractile performance in hearts affected by significant bioenergetic abnormalities and contractile dyskinesis (Feygin et al. 2007). Due to the low cell engraftment, the authors postulated that MSCs did not provide a structural contribution to the damaged heart but that the observed beneficial effects resulted from paracrine repair mechanisms.

It is known that myocardial infarction is associated with a strong inflammatory response; therefore, we can postulate that the paracrine effect exerted by MSCs is not just limited to cytoprotection or vascularization. MSCs produce and release molecules that limit local inflammation when injected into injured tissues. In vitro, the anti-inflammatory effect of MSCs is supported by the fact that adult rat cardiomyocytes (ARVCs) were injured when cultured in the presence of the monocyte chemoattractant protein (MCP-1) (related to myocarditis); in contrast, in the presence of MSC-CM, the MCP-1-induced injury was significantly attenuated (Fuse et al. 2001). Moreover, Ohnishi and colleagues confirmed that MSC transplantation into a rat model of acute myocarditis reduced $CD68^+$ inflammatory cells and MCP-1 expression and improved cardiac function (Ohnishi et al. 2007c).

Luan and colleagues showed that injection of MSCs in a rat model of pulmonary arterial hypertension resulted in an increased lung and heart function, reduced inflammation, and enhanced angiogenesis (Luan et al. 2012). MSC-treated groups showed a reduced expression of inflammatory mediators, such as interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), and matrix metallopeptidase-9 (MMP-9), but higher levels of vascular endothelial growth factor (VEGF) compared with untreated group, suggesting that these findings were mediated via secretion of anti-inflammatory mediators.

2.2 Renal Damage

Acute kidney injury (AKI) is a complex clinical syndrome that affects up to 20% of hospitalized patients. This syndrome is classically characterized by the rapid deterioration of renal function, with a subsequent accumulation of nitrogenous waste such as blood urea nitrogen (BUN) and creatinine and a decrease in urine output (Devarajan 2006). Although extensive efforts have been made to treat this disease, innovative interventions for AKI are needed (Lameire et al. 2013). For these reasons, it has been postulated that MSCs could exert a renoprotective effect for their trophic, anti-inflammatory, and pro-survival activity (Laflamme and Murry 2005; Little 2006; Aejaz et al. 2007). Togel and co-workers have also shown a renoprotective capacity of MSCs (Togel et al. 2005). Interestingly, although differentiation of injected MSCs was not detectable, the authors observed a significant reduction of pro-inflammatory cytokines (IL-1β, TNF-α, interferon-γ (IFN-γ), inducible nitric oxide synthase) and an upregulation of anti-inflammatory cytokines (IL-10, fibroblast growth factor (bFGF), TGF-α, Bcl-2) in AKI rats treated with BM-MSC infusion (Togel et al. 2005).

Kim and colleagues showed that the administration of human MSCs derived from adipose tissue (AD-MSCs) and the corresponding conditioned medium ameliorated

renal function and structure, increasing animal survival in an experimental model of AKI induced by cisplatin. MSCs were able to reduce renal tubular cell apoptosis and inflammation through paracrine mechanism (Kim et al. 2012). Furthermore, Morigi and colleagues demonstrated the cytoprotective effect of MSCs. Systemic infusion of MSCs, isolated from human umbilical cord blood, in a NOD/SCID mouse model of AKI was also able to reduce tubular apoptosis and prevent renal damage (Morigi et al. 2008). The vasculotropic, paracrine effect of rat BM-MSCs was also demonstrated in a study showing that MSC-CM containing VEGF, HGF, and IGF-1 improved aortic endothelial cell growth and survival (Togel et al. 2007). In contrast, in an I/R model of AKI, rats treated with VEGF-knockdown MSCs did not show any increase in renal microvessel density compared with MSC-treated rats (Togel et al. 2009).

MSCs secrete a number of factors, including VEGF, HGF, IGF-1, adrenomedullin, and stromal cell-derived factor 1 (SDF-1), that exert anti-apoptotic, mitogenic, vasoprotective, and angiogenic actions in AKI (Sun et al. 2019).

A pivotal role in kidney regeneration appears to be played by IGF-1, a growth factor described to have mitogenic effects on tubular cells (Hirschberg and Ding 1998). It has been reported that the knockdown of IGF-1 reduced tubular cell proliferation and increased apoptosis when MSCs were co-cultured with proximal tubular cells that had been damaged by cisplatin (Imberti et al. 2007). Furthermore, in mice with AKI, IGF-1 gene-silenced MSCs failed to exert their protective effect on renal function and tubular structure, thus indicating a key role of IGF-1 produced by MSCs in promoting regenerative processes in the kidney. Human cord blood MSCs (CB-MSCs) injected in mice with AKI can inhibit oxidative damage and induce the activation of the pro-survival gene Akt in tubular cells, thus promoting a pro-regenerative environment (Morigi et al. 2010). Notably, when co-cultured with proximal tubular cells, human CB-MSCs increased the levels of mitogenic and pro-survival factors including FGF, heparin-binding EGF-like growth factor, VEGF, and HGF in the cell supernatant and inhibited the release of IL-1β and TNF-α by proximal tubular cells exposed to cisplatin suggesting a regenerative and anti-inflammatory action of stem cell treatment (Rota et al. 2012). These observations suggest that MSCs may promote renal repair through the local release of mitogenic and vasculotropic factors.

2.3 Central Nervous System Diseases

Central nervous system (CNS) diseases are one of the most challenging and difficult conditions to address in terms of repair and regeneration. The treatments available for these conditions are largely ineffective, partly due to limitations in delivering the therapeutic agents to the brain. Recent advances in stem cell biology hold significant promise in the development of stem cell-based therapeutic strategies for neurodegenerative disorders (Bacigaluppi et al. 2009; Blurton-Jones et al. 2009; Pluchino et al. 2009; Barhum et al. 2010; Cusimano et al. 2012; Kokaia et al. 2012). Several groups have demonstrated that under specific culture conditions, MSCs develop neuronal morphologies in vitro, express neuronal markers (nestin, glial fibrillary

acidic protein, neuronal nuclear antigen, neuron specific enolase), and upregulate neuronal genes (Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Tondreau et al. 2008). Although controversial, these findings can be interpreted as the ability of MSCs to differentiate into neurons, hence suggesting potential for their use in cell replacement following CNS injury and disease. However, despite the expression of neuronal markers, many investigators failed to find evidence of neuronal differentiation in spinal cord or brain injury (Castro et al. 2002).

Growing evidence suggests that the improved neurological outcome observed may not be due to engraftment of MSCs at the lesion site and differentiation into neuronal cells (Cossetti et al. 2012; Lees et al. 2012; Pluchino and Cossetti 2013). At the contrary, severl groups have recently suggested that transplanted MSCs may be able to modulate the plasticity of damaged host tissues by secreting neurotrophic and survival-promoting growth factors, restoring synaptic transmitter release, reestablishing functional afferent/efferent connections, and exerting an immune modulatory effect (Uccelli et al. 2007, 2011; Siniscalco et al. 2010). For example, it has been shown that transplanted MSCs are able to induce survival and regeneration of host neurons (Crigler et al. 2006), increase proliferation of endogenous neural stem cells (Munoz et al. 2005), and increase the number of axons in a model of multiple sclerosis (Kassis et al. 2008).

The therapeutic effect observed after MSC treatment is mediated by secreted pro-survival factors such as IGF, HGF, VEGF, TGFß, FGF-2, brain-derived neurotrophic factor (BDNF), and platelet-derived growth factor-AB (Rehman et al. 2004; Constantin et al. 2009; Wei et al. 2009; Bai et al. 2012).

The intracerebroventricular administration of CM from MSCs resulted in reduced infarct volume in mice with I/R brain injury through a mechanism of neuroprotection that was dependent on secreted tissue inhibitor of TIMP-1 and progranulin (Egashira et al. 2012). The direct intravenous infusion of CM from MSCs derived from adipose tissue induced behavioral and learning recovery in rats with experimental hypoxia and ischemia brain injury while markedly reducing long-term functional cognitive and motor skill impairments through a mechanism regulated by secreted IGF-1 and BDNF (Wei et al. 2009).

When BM-MSCs are transplanted into the lesion epicenter of rats with experimental spinal cord injury (SCI), the BDNF and glial cell line-derived neurotrophic factor (GDNF) can reduce the lesion volume and induce axonal regrowth exerting a key role in neuronal protection (Gu et al. 2010). Furthermore, genetically modified MSCs overexpressing BDNF can reduce cell apoptosis and improve recovery from ischemia when injected into rats with transient middle cerebral artery occlusion (Kurozumi et al. 2004).

Therapies using BDNF have been applied in the treatment of Huntington's disease (HD), a genetic inherited progressive brain disorder that causes uncontrolled movements, emotional problems, and loss of thinking ability. Zuccato and colleagues reported that HD patients have lower levels of BDNF due to inhibition at transcriptional level by the mutant huntingtin protein (Zuccato et al. 2011). This reduction in BDNF in the striatum correlates with symptom onset and heightened severity of the disease in transgenic HD mice. MSCs engineered to overexpress

BDNF resulted in motor improvement and increased neuron viability when transplanted in HD mice (Dey et al. 2010; Pollock et al. 2016).

It has also been shown that MSCs secrete soluble factors (BDNF, FGF-2, GDNF, and IGF-1) that play an important role in promoting the neuronal survival in Parkinson's disease (PD) and SCI (McCoy et al. 2008; Lopatina et al. 2011). PD is a chronic and progressive neurologic disease linked to decreased dopamine production in the substantia nigra which is responsible for muscle tremors, rigidity, slowness of movement, and impaired balance. Shintani et al. demonstrated that MSC-mediated secretion of BDNF, GDNF, and bFGF promoted survival of dopaminergic neurons in rat primary culture of ventral mesencephalic cells (Shintani et al. 2007). Similarly, another group demonstrated that the injection of MSCs in 6-hydroxydopamine-induced lesioned rat model regenerates the damaged striatal dopaminergic nerve and increased dopamine levels through the release of BDNF and GDNF (Sadan et al. 2009).

SCI is a long-term functional deficit that leads to progressive loss of neurons, glial cells, inflammation, demyelination, and pain. In this case, cellular therapy aims to reconstruct the spinal cord through cellular replacement, glial scar remodeling, axonal guidance, and filling of formed syringomyelia. Studies by Hofstetter et al. (2002) indicated that transplanted MSCs attenuate acute inflammation and promote functional recovery following SCI. Ohta et al. (2004) suggested that BM-MSCs reduced post-SCI cavity formation and improved behavioral function by releasing trophic factors into the cerebrospinal fluid (CSF) or by direct interaction with host spinal tissues. Other studies demonstrated that transplanted BM-MSCs promote axonal growth, glial scar reduction, and neurite outgrowth through the release of BDNF, nerve growth factor, and neurotrophin-3 (Crigler et al. 2006). Similar results were reported by Neuhuber et al. who reported that the BM-MSC-CM promotes axon growth and functional recovery through the effect of BDNF, VEGF, IL-6, monocyte chemoattractant protein-1, and SDF-1 alpha (Lu et al. 2005; Neuhuber et al. 2005).

2.4 Wound Healing and Peripheral Artery Disease

Wound healing is a dynamic process consisting of continuous, overlapping, and precisely programmed phases. The events of each phase occur in a temporal and coordinated manner. Interruption or modulation of the process can lead to delayed wound healing or a non-healing chronic wound. Impairment of normal wound healing processes often occurs in diabetic patients, who can develop gangrenous lesions or diabetic ulcers, often requiring amputation. Advancement of any regenerative therapy to repair such wounds and to promote fast healing would be clinically very useful. Recently, there has been much interest in the development of MSC-based therapies for cutaneous wounds apparently able to promote both rapid closure and healing without scar formation.

For instance, it has been demonstrated that MSC administration in experimental rodent models of acute or diabetic wounds can facilitate healing, accelerating

epithelialization and increasing granulation tissue formation and angiogenesis (Li et al. 2006; McFarlin et al. 2006; Javazon et al. 2007; Wu et al. 2007; Alfaro et al. 2008; Sasaki et al. 2008). There is emerging evidence indicating that MSC paracrine signaling is the main mechanism responsible for enhanced wound repair. Numerous studies observed that MSC-CM exerts the same effect as MSCs on wound repair acting as a chemoattractant for epidermal keratinocytes, dermal fibroblasts, macrophages, and endothelial cells (Javazon et al. 2007; Kim et al. 2007; Chen et al. 2008; Lee et al. 2009; Smith et al. 2010). Furthermore, it has been shown that dermal fibroblasts secrete increased amounts of collagen type I (Kim et al. 2007) and alter gene expression in response to either MSCs or MSC-CM (Smith et al. 2010). These in vitro results were supported by the identification of high levels of growth factors and chemokines known to promote wound healing including epidermal growth factor (EGF), keratinocyte growth factor, IGF-1, VEGF-α, erythropoietin, SDF-1, macrophage inflammatory protein (MIP)-1a, and MIP-1b (Chen et al. 2008). Recently, it has also been reported that conditioned medium from aorta-derived $CD133^+$ progenitor cells could promote and accelerate healing of diabetic ischemic ulcers through the release of cytokines able to stimulate endothelial cell proliferation, migration, and survival (Barcelos et al. 2009). These various studies suggest that the MSCs may act in a paracrine fashion in the wound healing process.

Peripheral artery disease (PAD) is mainly caused by atherosclerosis that narrows and blocks arteries in various critical regions of the body. The most common symptoms of PAD involving the lower extremities are cramping, chronic wounds, and ulcers followed by gangrene and amputation. Thanks to their angiogenic potential, MSCs can be considered effective potential treatment option since restoration of the blood flow might eventually restore the functionality of the affected extremity (Kinnaird et al. 2004; Iwase et al. 2005; Moon et al. 2006; Hung et al. 2007; Wu et al. 2007; Chen et al. 2008; Boomsma and Geenen 2012). In a murine model of hindlimb ischemia, local intramuscular injection of BM-MSCs increased blood vessel density and improved perfusion reducing the incidence of auto-amputation. These results occurred without MSC incorporation into the blood vessels, suggesting that increased levels of VEGF and FGF-2 were probably mediated by the paracrine actions of the MSCs (Kinnaird et al. 2004). Other studies showed that transplanted MSCs could differentiate into endothelial cells (Iwase et al. 2005; Moon et al. 2006), even though incorporation rates into the host vascular structures were very limited indicating that the paracrine effects mainly account for the beneficial effects observed (Moon et al. 2006). In this context, several studies have demonstrated that the MSC secretome includes a broad range of pro-angiogenic factors including VEGF, FGF-2, angiopoietin-1, MCP-1, interleukin-6 (IL-6), and placental growth factor (Kinnaird et al. 2004; Hung et al. 2007; Wu et al. 2007; Chen et al. 2008; Boomsma and Geenen 2012).

A phase II clinical trial demonstrated that intra-arterial infusion of autologous BM cells significantly increased both peripheral blood perfusion and the percentage of amputation-free patients with critical limb ischemia (CLI) 1 year after the treatment in patients with severe limb ischemia (Schiavetta et al. 2012). The effects of a combination of mononuclear cells from BM (BM-MNCs) and BM-MSCs were

also tested. Regardless of dosage, patients receiving cell injection showed significant improvements in ankle-brachial indices (ABI), walking time, pain, and limb perfusion, and significant wound healing (Lasala et al. 2012). The clinical trial performed by Lu and colleagues directly evaluated the effects of intramuscular injections of ex vivo-expanded autologous BM-MSCs in comparison with BM-MNCs (Lu et al. 2011). In 41 type II diabetic patients with bilateral CLI, a significant improvement in ABI, pain, walking time, wound healing, and collateral artery enlargement was observed after administration of both BM-MNCs and BM-MSCs. Enhanced effects were reported in the BM-MSC group. Given the observation of treatment with MSCs indicating the potential for improved blood flow recovery in patients (Iwase et al. 2005; Lu et al. 2011), a number of new clinical trials utilizing MSCs are underway (Gnecchi et al. 2012; Samura et al. 2017).

2.5 MSC-Based Therapy for Liver Fibrosis Treatment

Liver fibrosis is defined as the excessive accumulation of extracellular matrix proteins that occurs after chronic injury. Injuries may result from viral hepatitis, autoimmune attack, drugs, alcohol, or metabolic disease. Although fibrosis is asymptomatic, its progression to cirrhosis leads to morbidity and mortality (Friedman 2003).

The most effective treatment for cirrhosis is liver transplantation (Fallowfield and Iredale 2004); however, there are obvious limitations including a shortage of donor organs, surgical complications, immunological rejection, and high medical costs (Dutkowski et al. 2015). A possible alternative approach to liver transplantation is represented by application of cell-based hepatocyte therapy. A decrease in liver fibrosis and restoration of phospholipid secretion were observed in a mouse model of progressive familial intrahepatic cholestasis type III after hepatocyte transplantation (De Vree et al. 2000). Unfortunately, the low cell viability and instability of transplantable hepatocytes have hampered their clinical application. Studies have shown that less than 30% of transplanted hepatocytes survive in vivo. Moreover, the surviving cells have limited replicative potential and reduced hepatic function as a result of culture in vitro (Forbes and Alison 2014; Komori et al. 2014). Alternative cell sources for liver treatment have been identified in embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), hematopoietic stem cells, and MSCs that can be differentiated into hepatocyte-like cells both in vitro and in vivo (Shu et al. 2004; Si-Tayeb et al. 2010; Gnecchi et al. 2017). Although many studies showed that ESCs injected into CCL4-injured mice efficiently differentiate into hepatocytes (Yamamoto et al. 2003; Moriya et al. 2007) and reduce fibrosis, Choi and colleagues reported that injection of undifferentiated mouse ESCs into the spleen of immunosuppressed nude mice can result in teratoma formation (Choi et al. 2002). More recently, it has been shown that iPSC can differentiate into hepatocytes when injected in experimental model of liver fibrosis (Liu et al. 2011). Nevertheless, before clinical application many issue regarding genetic manipulation and mutagenesis should be clarified.

Interestingly, MSCs have shown relevant therapeutic effects in experimental models of liver fibrosis. Recent publications have shown that MSCs can be

differentiated into hepatocyte-like cells in the presence of chemical compounds or specific growth factors such as HGF, oncostatin M (OSM), EGF, TGF-β, bFGF, IGF, dexamethasone, and nicotinamide (Forte et al. 2006; Snykers et al. 2006). Although MSC differentiation into hepatocytes has been demonstrated in vitro, it remains highly controversial whether MSC transplantation can regenerate hepatocytes in vivo. The vast majority of recently published studies indicate that the therapeutic effects observed after MSC injection, in both acute and chronic liver failure models, are likely mediated by the release of trophic and immunomodulatory factors (Ishikawa et al. 2007; Kharaziha et al. 2009; Jang et al. 2014).

Following liver injury, hepatic stellate cells (HSC) were activated into proliferative, α-smooth muscle actin-positive, myofibroblast-like, and extracellular matrix-producing cells (Friedman 2008). Several in vitro studies have demonstrated the ability of MSCs to indirectly modulate HSC activation via paracrine mechanisms. Using indirect co-culture systems, Parekkadan et al. showed that human BM-MSCs can inhibit collagen synthesis in activated rat HSC (Parekkadan et al. 2007). Moreover, MSCs inhibited HSC proliferation, even if HSC did not revert to a quiescent state. The modulation of HSC activity was attributed to the presence of IL-10, TNF-α, and HGF secreted by the MSCs.

Other studies reported that BM-MSC-conditioned medium exerts anti-apoptotic and pro-mitotic effects on cultured hepatocytes. For instance, van Poll et al. provided the first evidence that delivery of MSC-CM can dramatically reduce cell death and enhance liver regeneration in D-galactosamine-induced fulminant hepatic failure in vivo and in vitro (van Poll et al. 2008). Authors reported that MSC-CM therapy led to 90% reduction in apoptotic hepatocytes and a threefold increase in the number of proliferating hepatocytes in vivo. Kim (SDF-1, HGF, IGF-1, VEGF), (EGF, HGF, NGF, TGF-α), (VEGF) (Kim et al. 2012). Things become more challenging when considering that paracrine factors of human fetal MSCs might inhibit liver cancer growth, a tumor-specific, antiproliferative effect that is not observed with normal human hepatocyte cells (Yulyana et al. 2015). Experimental evidences have revealed that soluble factors secreted by MSCs protect hepatocytes and reduce pro-inflammatory events responsible for liver fibrosis providing a protection against chronic liver injury (Cui et al. 2014). Zhao et al. observed an increase in the level of anti-inflammatory cytokines such as IL-10 and decreased levels of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and TGF-β in mice treated with intravenous infusion of MSCs (Zhao et al. 2012). Based on these experimental results, it is possible to hypothesize that MSCs may represent a clinically relevant solution for the treatment of liver fibrosis; however, a significant number of issues remain to be clarified before MSCs can be proposed as a therapeutic option to treat liver fibrosis.

3 Inside the MSC Secretome: An Overview

A growing body of works supports the possibility to repair damaged tissue by harnessing the reparative potential of paracrine factors instead of using whole cells, introducing a different dimension to the therapeutic applications of MSCs in

regenerative medicine. There is a body of work suggesting the MSC-derived secretome plays a key role in a number of biological functions (differentiation, apoptosis, angiogenesis, or signaling) as evidenced by studies performed in the setting of cardiovascular, renal, liver, and neurodegenerative diseases (Kuo et al. 2008; Mirotsou et al. 2011; Uccelli et al. 2011; Cantaluppi et al. 2013; Danieli et al. 2016). A cell-free-based approach may be more easily translated to the clinical arena for obvious reasons. A secretome-based approach would bypass various issues associated with cell-based therapy (i.e., immune compatibility, tumorigenicity, xenozootic infections, waiting time for ex vivo expansion of autologous cell preparations) and lead to therapies with more predictable outcomes. However, the study of MSC secretome is challenging given the difficulties in collection and preparation of small quantities of the secreted proteins. In addition, culture media are rich in salts and other compounds that interfere with most proteomic techniques. Furthermore, the presence of serum proteins dramatically influences the dynamic range of the sample and, thereby, the identification of secreted proteins. Recent improvements in stem cell culture techniques and technological advancements in the field of proteomics have significantly facilitated secretome analysis. Proteomic investigation of the secretome is currently based on conventional techniques such as multiplex antibody-based techniques. These assays (e.g., RayBio cytokine arrays or Luminex®) offer high sensitivity (typically 1–10 pg/ml) as well as high specificity and reproducibility across a broad range of concentrations and the potential for high-throughput analysis. Using this technique, Nguyen et al. were able to identify the MSC-derived factors (i.e., VEGF, endothelin, epiregulin, galectin-3, SMAD5, secreted frizzled-related protein (sRFP)-1, and sRFP-4) involved in cardiac improvement in a swine MI model (Nguyen et al. 2010). Other commonly used proteomic approaches are based on protein/peptide separation (2-D or 2-D fluorescence difference gel electrophoresis) followed by mass spectrometry, enzyme-linked immunosorbent assay (ELISA), and subsequent validation using Western blot approaches. However, the highly bioactive molecules secreted are often difficult to identify using conventional gel-based techniques. More specific techniques like the stable isotope labeling by amino acids in cell culture (SILAC – based on the incorporation of nonradioactive-labelled arginine/lysine into the newly synthesized proteins) or isobaric tag for relative and absolute quantitation (iTRAQ) or isotope-coded affinity tag (ICAT) labeling are often used in association with gel-independent techniques and 1-D or 2-D liquid chromatography-tandem mass spectrometer (LC-MS/MS) for thorough characterization of the secretome (Skalnikova et al. 2011). Using LC-MS/MS technique, Estrada et al. were able to profile the proteome of murine MSCs and identified 258 proteins, 54 of which were classified as secreted proteins (Estrada et al. 2009). Sarojini et al. applied the LC-MS/MS approach to MSC-CM and identified 19 secreted proteins including extracellular matrix structural proteins, collagen processing enzymes, pigment epithelium-derived factor, and cystatin C (Sarojini et al. 2008). Lee and colleagues used LC-MS/MS to analyze the secretome of human adipose tissue-derived MSCs preconditioned with TNF-α and identified 187 secreted proteins (Lee et al. 2010). Interestingly, 118 out of these 187 proteins, including IL-6, IL-8, MCP-1, MMPs, pentraxin-related protein 3, and cathepsin L,

were secreted at higher levels following MSC exposure to TNF-α (Lee et al. 2010). Despite recent advances in the characterization of the MSC secretome, current techniques have only proved efficacious in the identification of factors expressed at high levels (gel-based and LC-MS/MS have limited sensitivity in low molecule concentration detection, and antibody-based techniques are limited by the availability of antibodies). Hence, the developments of new approaches are necessary to directly quantify and characterize the full profile of secreted factors.

A better understanding of the MSC secretome will help to define which factors are involved in the therapeutic effects of stem cells. Recent studies suggest that besides their ability to release soluble factors in the microenvironment, MSCs are able to produce more complex structures called microvesicles (MVs) and exosomes (Exo), responsible for the intercellular communication between MSCs and target cells (Baglio et al. 2012; Tomasoni et al. 2013). MVs and Exo have attracted the attention of the research community given their potential beneficial influence, comparable to the regenerative effects obtained with stem cell transplantation in several preclinical disease models (Camussi et al. 2013).

Distinction between MVs and Exo is based on size and isolation methodologies as MVs and Exo are structurally and morphologically distinct. MVs are generated from the budding of the cell membrane, contain cytoplasmic materials, and are heterogeneous in size (100–1000 nm diameter). MVs are secreted by a variety of stem and somatic cells, either constitutively or when specifically stimulated (i.e. apoptosis); MVs are also present in most body fluids and in interstitial space between cells (Lai et al. 2011; Schifferli 2011). In contrast, Exo are nanoparticles (40–100 nm) derived from an endocytosis process within cells and have better defined biophysical and biochemical properties compared with MVs (Dragovic et al. 2011). The Exo membrane is enriched in cholesterol, sphingomyelin, and ceramide and contains lipid rafts; they have a specific flotation density in sucrose of 1.13–1.19 g/ml, sediment at 100,000 g (Thery et al. 2009), and express specific markers such as CD81, CD63, and CD9 as well as cell type-specific antigens derived from the parental cell they originate from (Mathivanan et al. 2010). Exo contain proteins, growth factors, mRNA, and microRNA that can be transferred to recipient cells modulating gene expression and promoting therapeutic responses.

MVs and Exo produced by MSCs have been associated with repairative process in a number of preclinical models, including liver fibrosis, stroke, acute lung injury, acute kidney injury, and cardiovascular diseases (Lai et al. 2010, 2013; Gatti et al. 2011; Sdrimas and Kourembanas 2014; Tomasoni et al. 2013; Xin et al. 2013).

Bruno and colleagues demonstrated that MVs released by MSCs exerted proliferative and anti-apoptotic effects on tubular epithelial cells both in vitro and in vivo when delivered in a mouse model of AKI (Bruno et al. 2009). The renal repair properties were attributed to the transfer of specific mRNAs encoding for proteins responsible for the control of cell proliferation, transcription, and immune response. In fact, when MSC-MVs were treated with RNAse prior injection, their reparative effect was abolished (Cantaluppi et al. 2012). Tomasoni and colleagues documented that MVs and Exo released by human BM-MSC induce proliferation of proximal

tubular cells damaged by cisplatin through the transfer of IGF-1R mRNA that was translated into its corresponding protein (Tomasoni et al. 2013).

A recent study reported that the beneficial effect observed following MSC-CM treatment in myocardial I/R injury was mediated by small molecules of 100–200 nm size (Timmers et al. 2007). The therapeutic effect of MVs was further confirmed in a mouse model of myocardial I/R injury, showing that the treatment with MSC-Exo resulted in restoration of bioenergetics, reduction of oxidative stress, and activation of pro-survival signaling pathways, overall enhancing the cardiac function (Arslan et al. 2013). Yu and colleagues showed that MSCs overexpressing GATA-4 enhanced cytoprotection by reducing the levels of p53 in the treated cells via the MV-mediated transfer of the anti-apoptotic miR-22 (Yu et al. 2013). It is also known that MSC-CM can stimulate proliferation of endothelial cells both in vitro and in vivo. In addition to the involvement of pro-angiogenic cytokines such as VEGF and HGF-1, it has been demonstrated that MVs collected from hypoxia-preconditioned MSC-CM can be internalized by endothelial cells and promote cell proliferation (Zhang et al. 2012). In rats with traumatic brain injury (TBI), the administration of MSC-derived Exo significantly increased the number of newly generated endothelial cells in the lesion border zone and the number of newly formed immature and mature neurons in the dentate gyrus and reduced neuroinflammation (Zhang et al. 2015). Tan and colleagues showed that MSC-derived Exo can elicit a hepato-protective effect against toxicant-induced injury, mainly through the activation of proliferative and regenerative responses (Tan et al. 2014). Furthermore, in a model of fibrotic liver injury, the administration of MSC-derived Exo inhibited collagen deposition and preserved liver function in a manner similar to the administration of MSCs (Li et al. 2013). Although these results suggest that MSC-derived Exo may have significant therapeutic potential, further understanding of their nature, content, and mechanism of action needs to be investigated.

4 Conclusions

Over the last decade, MSCs have garnered significant attention in regenerative medicine, and several clinical trials are ongoing to assess their therapeutic potential for the treatment of a number of conditions. At the same time, the discovery that the beneficial effects documented after MSC-based therapies are mainly mediated by their secretome has offered a new avenue in the field of cell therapy. Advances in proteomic technology, protein arrays, and bioinformatics have facilitated secretome analysis; however, uncovering the molecular and biochemical pathways mediating the MSC paracrine effects is essential to better elucidate the secretome's profile and its potential clinical use. The use of MSC-derived secretome represents a novel and promising alternative to cell-based therapy, with the potential to address several concerns and risks associated with direct use of stem cells (Gnecchi 2018). However, a number of limitations in the application of protein therapy needs to be addressed to fully exploit the potential of this therapeutic approach, including issues around therapeutic protein concentrations, protein stability, pharmacokinetics, and

persistency of the therapeutic effect. Although the road to optimal protein therapy remains challenging with a number of significant hurdles, advances in experimental design and proteomic technologies offer the opportunity for significant improvements in the efficacy and safety of protein therapies, setting the basis for new regenerative medicine approaches.

Acknowledgments This work was supported by the Ministero Italiano della Sanità [Grant number GR-2008-1142871 and GR-2010-2320533], the Fondazione Cariplo [Grant number 2007-5984], and the Ministero Italiano degli Affari Esteri [Grant number ZA11GR2].

References

Aejaz HM, Aleem AK, Parveen N, Khaja MN, Narusu ML, Habibullah CM (2007) Stem cell therapy-present status. Transplant Proc 39:694–699

Alfaro MP, Pagni M, Vincent A, Atkinson J, Hill MF, Cates J, Davidson JM, Rottman J, Lee E, Young PP (2008) The Wnt modulator sFRP2 enhances mesenchymal stem cell engraftment, granulation tissue formation and myocardial repair. Proc Natl Acad Sci U S A 105:18366–18371

Arslan F, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, Timmers L, van Rijen V, Doevendans PA, Pasterkamp G, Lim SK, de Kleijn DP (2013) Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. Stem Cell Res 10:301–312

Bacigaluppi M, Pluchino S, Peruzzotti-Jametti L, Kilic E, Kilic U, Salani G, Brambilla E, West MJ, Comi G, Martino G, Hermann DM (2009) Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. Brain 132:2239–2251

Baglio SR, Pegtel DM, Baldini N (2012) Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. Front Physiol 3:359

Bai L, Lennon DP, Caplan AI, DeChant A, Hecker J, Kranso J, Zaremba A, Miller RH (2012) Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. Nat Neurosci 15:862–870

Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, Siragusa M, Meloni M, Campesi I, Monica M et al (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

Barhum Y, Gai-Castro S, Bahat-Stromza M, Barzilay R, Melamed E, Offen D (2010) Intracerebroventricular transplantation of human mesenchymal stem cells induced to secrete neurotrophic factors attenuates clinical symptoms in a mouse model of multiple sclerosis. J Mol Neurosci 41:129–137

Berry MF, Engler AJ, Woo YJ, Pirolli TJ, Bish LT, Jayasankar V, Morine KJ, Gardner TJ, Discher DE, Sweeney HL (2006) Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am J Physiol Heart Circ Physiol 290:H2196–H2203

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

Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2:313–319

Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang C-Y (2013) The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19:35–42

Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller 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 U S A 106:13594–13599

Boomsma RA, Geenen DL (2012) Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. PLoS One 7:e35685

Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B, Tetta C, Camussi G (2009) Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. J Am Soc Nephrol 20:1053–1067

Camussi G, Deregibus MC, Cantaluppi V (2013) Role of stem-cell-derived microvesicles in the paracrine action of stem cells. Biochem Soc Trans 41:283–287

Cantaluppi V, Gatti S, Medica D, Figliolini F, Bruno S, Deregibus MC, Sordi A, Biancone L, Tetta C, Camussi G (2012) Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells. Kidney Int 82:412–427

Cantaluppi V, Biancone L, Quercia A, Deregibus MC, Segoloni G, Camussi G (2013) Rationale of mesenchymal stem cell therapy in kidney injury. Am J Kidney Dis 61:300–309

Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD (2002) Failure of bone marrow cells to transdifferentiate into neural cells in vivo. Science 297:1299

Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M (2001) Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke 32:1005–1011

Chen L, Tredget EE, Wu PY, Wu Y (2008) Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 3:e1886

Choi D, Oh HJ, Chang UJ, Koo SK, Jiang JX, Hwang SY, Lee JD, Yeoh GC, Shin HS, Lee JS, Oh B (2002) In vivo differentiation of mouse embryonic stem cells into hepatocytes. Cell Transplant 11:359–368

Ciuffreda MC, Malpasso G, Musarò P, Turco V, Gnecchi M (2016) Protocols for in vitro differentiation of human mesenchymal stem cells into osteogenic chondrogenic and adipogenic lineages. Methods Mol Biol 1416:149–158

Ciuffreda MC, Malpasso G, Chokoza C, Bezuidenhout D, Goetsch KP, Mura M, Pisano F, Davies NH, Gnecchi M (2018) Synthetic extracellular matrix mimic hydrogel improves efficacy of mesenchymal stromal cell therapy for ischemic cardiomyopathy. Acta Biomater 70:71–83

Constantin G, Marconi S, Rossi B, Angiari S, Calderan L, Anghileri E, Gini B, Bach SD, Martinello M, Bifari F et al (2009) Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. Stem Cells 27:2624–2635

Cossetti C, Alfaro-Cervello C, Donega M, Tyzack G, Pluchino S (2012) New perspectives of tissue remodelling with neural stem and progenitor cell-based therapies. Cell Tissue Res 349:321–329

Crigler L, Robey RC, Asawachaicharn A, Gaupp D, Phinney DG (2006) Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. Exp Neurol 198:54–64

Cui L, Shi Y, Han Y, Fan D (2014) Immunological basis of stem cell therapy in liver diseases. Expert Rev Clin Immunol 10:1185–1196

Cusimano M, Biziato D, Brambilla E, Donega M, Alfaro-Cervello C, Snider S, Salani G, Pucci F, Comi G, Garcia-Verdugo JM et al (2012) Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. Brain 135:447–460

Danieli P, Malpasso G, Ciuffreda MC, Cervio E, Calvillo L, Copes F, Pisano F, Mura M, Kleijn L, de Boer RA et al (2015) Conditioned medium from human amniotic mesenchymal stromal cells limits infarct size and enhances angiogenesis. Stem Cells Transl Med 4:448–458

Danieli P, Malpasso G, Ciuffreda MC, Gnecchi M (2016) Testing the paracrine properties of human mesenchymal stem cells using conditioned medium. Methods Mol Biol 1416:445–456

De Vree JM, Ottenhoff R, Bosma PJ, Smith AJ, Aten J, Oude Elferink RP (2000) Correction of liver disease by hepatocyte transplantation in a mouse model of progressive familial intrahepatic cholestasis. Gastroenterology 119:1720–1730

Devarajan P (2006) Update on mechanisms of ischemic acute kidney injury. J Am Soc Nephrol 17:1503–1520

Dey ND, Bombard MC, Roland BP, Davidson S, Lu M, Rossignol J, Sandstrom MI, Skeel RL, Lescaudron L, Dunbar GL (2010) Genetically engineered mesenchymal stem cells reduce

behavioral deficits in the YAC 128 mouse model of Huntington's disease. Behav Brain Res 214:193–200
Dimmeler S, Zeiher AM, Schneider MD (2005) Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 115:572–583
Ding DC, Shyu WC, Lin SZ (2011) Mesenchymal stem cells. Cell Transplant 20:5–14
Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ, Hole P, Carr B, Redman CW, Harris AL, Dobson PJ, Harrison P, Sargent IL (2011) Sizing and phenotyping of cellular vesicles using nanoparticle tracking analysis. Nanomedicine 7:780–788
Du T, Cheng J, Zhong L, Zhao XF, Zhu J, Zhu YJ, Liu GH (2012) The alleviation of acute and chronic kidney injury by human Wharton's jelly-derived mesenchymal stromal cells triggered by ischemia-reperfusion injury via an endocrine mechanism. Cytotherapy 14:1215–1227
Duerr RL, Huang S, Miraliakbar HR, Clark R, Chien KR, Ross J Jr (1995) Insulin-like growth factor-1 enhances ventricular hypertrophy and function during the onset of experimental cardiac failure. J Clin Invest 95:619–627
Duffield JS, Park KM, Hsiao LL, Kelley VR, Scadden DT, Ichimura T, Bonventre JV (2005) Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. J Clin Invest 115:1743–1755
Dutkowski P, Linecker M, DeOliveira ML, Mullhaupt B, Clavien PA (2015) Challenges to liver transplantation and strategies to improve outcomes. Gastroenterology 148:307–323
Egashira Y, Sugitani S, Suzuki Y, Mishiro K, Tsuruma K, Shimazawa M, Yoshimura S, Iwama T, Hara H (2012) The conditioned medium of murine and human adipose-derived stem cells exerts neuroprotective effects against experimental stroke model. Brain Res 1461:87–95
Erices A, Conget P, Minguell JJ (2000) Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 109:235–242
Estrada R, Li N, Sarojini H, An J, Lee MJ, Wang E (2009) Secretome from mesenchymal stem cells induces angiogenesis via Cyr61. J Cell Physiol 219:563–571
Fallowfield JA, Iredale JP (2004) Targeted treatments for cirrhosis. Expert Opin Ther Targets 8:423–435
Fang TC, Pang CY, Chiu SC, Ding DC, Tsai RK (2012) Renoprotective effect of human umbilical cord-derived mesenchymal stem cells in immunodeficient mice suffering from acute kidney injury. PLoS One *7*:e46504
Feygin J, Mansoor A, Eckman P, Swingen C, Zhang J (2007) Functional and bioenergetic modulations in the infarct border zone following autologous mesenchymal stem cell transplantation. Am J Physiol Heart Circ Physiol 293:H1772–H1780
Forbes SJ, Alison MR (2014) Regenerative medicine. Knocking on the door to successful hepatocyte transplantation. Nat Rev Gastroenterol Hepatol 11:277–278
Forte G, Minieri M, Cossa P, Antenucci D, Sala M, Gnocchi V, Fiaccavento R, Carotenuto F, De Vito P, Baldini PM, Prat M, Di Nardo P (2006) Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. Stem Cells 24:23–33
Freestone NS, Ribaric S, Mason WT (1996) The effect of insulin-like growth factor-1 on adult rat cardiac contractility. Mol Cell Biochem 163–164:223–229
Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP (1968) Heterotopic of bone marrow. Anal Pecursor Cells Osteogenic Hematopoietic Tissues Transplan 6:230–247
Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393–403
Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 2:83–92
Friedman SL (2003) Liver fibrosis – from bench to bedside. J Hepatol 38(Suppl 1):S38–S53
Friedman SL (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 88:125–172
Fuse K, Kodama M, Hanawa H, Okura Y, Ito M, Shiono T, Maruyama S, Hirono S, Kato K, Watanabe K, Aizawa Y (2001) Enhanced expression and production of monocyte chemoattractant protein-1 in myocarditis. Clin Exp Immunol 124:346–352

Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, Camussi G (2011) Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. Nephrol Dial Transplant 26:1474–1483

Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11:367–368

Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20:661–669

Gnecchi M, Zhang Z, Ni A, Dzau VJ (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103:1204–1219

Gnecchi M, Danieli P, Cervio E (2012) Mesenchymal stem cell therapy for heart disease. Vasc Pharmacol 57:48–55

Gnecchi M, Danieli P, Malpasso G, Ciuffreda MC (2016) Paracrine mechanisms of mesenchymal stem cells in tissue repair. Methods Mol Biol 1416:123–146

Gnecchi M, Stefanello M, Mura M (2017) Induced pluripotent stem cell technology: toward the future of cardiac arrhythmias. Int J Cardiol 237:49–52

Gnecchi M (2018) Cell therapy for heart regeneration: Learning from the past to build a brighter future. Stem Cells Transl Med 7(10):702–704. https://doi.org/10.1002/sctm.18-0126. Epub 2018 Sep 8

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM (2001) Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189:54–63

Gu W, Zhang F, Xue Q, Ma Z, Lu P, Yu B (2010) Transplantation of bone marrow mesenchymal stem cells reduces lesion volume and induces axonal regrowth of injured spinal cord. Neuropathology 30:205–217

Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE et al (2010) Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 107:913–922

Hirschberg R, Ding H (1998) Mechanisms of insulin-like growth factor-I-induced accelerated recovery in experimental ischemic acute renal failure. Miner Electrolyte Metab 24:211–219

Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci U S A 99:2199–2204

Hung SC, Pochampally RR, Chen SC, Hsu SC, Prockop DJ (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 25:2363–2370

Imberti B, Morigi M, Tomasoni S, Rota C, Corna D, Longaretti L, Rottoli D, Valsecchi F, Benigni A, Wang J et al (2007) Insulin-like growth factor-1 sustains stem cell mediated renal repair. J Am Soc Nephrol 18:2921–2928

In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FH, Willemze R, Fibbe WE, Kanhai HH (2003) Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 102:1548–1549

Ishikawa T, Terai S, Urata Y, Marumoto Y, Aoyama K, Murata T, Mizunaga Y, Yamamoto N, Nishina H, Shinoda K, Sakaida I (2007) Administration of fibroblast growth factor 2 in combination with bone marrow transplantation synergistically improves carbon-tetrachloride-induced liver fibrosis in mice. Cell Tissue Res 327:463–470

Iso Y, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, Sobel BE, Delafontaine P, Prockop DJ (2007) Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun 354:700–706

Iwase T, Nagaya N, Fujii T, Itoh T, Murakami S, Matsumoto T, Kangawa K, Kitamura S (2005) Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc Res 66:543–551

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312

Jang YO, Kim YJ, Baik SK, Kim MY, Eom YW, Cho MY, Park HJ, Park SY, Kim BR, Kim JW et al (2014) Histological improvement following administration of autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: a pilot study. Liver Int 34:33–41

Javazon EH, Keswani SG, Badillo AT, Crombleholme TM, Zoltick PW, Radu AP, Kozin ED, Beggs K, Malik AA, Flake AW (2007) Enhanced epithelial gap closure and increased angiogenesis in wounds of diabetic mice treated with adult murine bone marrow stromal progenitor cells. Wound Repair Regen 15:350–359

Jiang S, Haider HK, Idris NM, Salim A, Ashraf M (2006) Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. Circ Res 99:776–784

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272

Jung KH, Shin HP, Lee S, Lim YJ, Hwang SH, Han H, Park HK, Chung JH, Yim SV (2009) Effect of human umbilical cord blood-derived mesenchymal stem cells in a cirrhotic rat model. Liver Int 29:898–909

Kassis I, Grigoriadis N, Gowda-Kurkalli B, Mizrachi-Kol R, Ben-Hur T, Slavin S, Abramsky O, Karussis D (2008) Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. Arch Neurol 65:753–761

Keyser KA, Beagles KE, Kiem HP (2007) Comparison of mesenchymal stem cells from different tissues to suppress T-cell activation. Cell Transplant 16:555–562

Kharaziha P, Hellstrom PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M, Atashi A, Honardoost M, Zali MR, Soleimani M (2009) Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial. Eur J Gastroenterol Hepatol 21:1199–1205

Kim WS, Park BS, Sung JH, Yang JM, Park SB, Kwak SJ, Park JS (2007) Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. J Dermatol Sci 48:15–24

Kim JH, Park DJ, Yun JC, Jung MH, Yeo HD, Kim HJ, Kim DW, Yang JI, Lee GW, Jeong SH, Roh GS, Chang SH (2012) Human adipose tissue-derived mesenchymal stem cells protect kidneys from cisplatin nephrotoxicity in rats. Am J Physiol Ren Physiol 302:F1141–F1150

Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109:1543–1549

Kokaia Z, Martino G, Schwartz M, Lindvall O (2012) Cross-talk between neural stem cells and immune cells: the key to better brain repair? Nat Neurosci 15:1078–1087

Komori J, DeWard AD, Gramignoli R, Strom SC, Fontes P, Lagasse E (2014) Potential barriers to human hepatocyte transplantation in MUP-uPAtg((+)/(+))Rag2(-)/(-)gammaC(-)/(-) mice. Cell Transplant 23:1537–1544

Kuo TK, Hung SP, Chuang CH, Chen CT, Shih YR, Fang SC, Yang VW, Lee OK (2008) Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells. Gastroenterology 134:2111–2121.e1-3

Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Kobune M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K et al (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

Laflamme MA, Murry CE (2005) Regenerating the heart. Nat Biotechnol 23:845–856

Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM et al (2010) Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 4:214–222

Lai RC, Chen TS, Lim SK (2011) Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regen Med 6:481–492

Lameire NH, Bagga A, Cruz D, De Maeseneer J, Endre Z, Kellum JA, Liu KD, Mehta RL, Pannu N, Van Biesen W, Vanholder R (2013) Acute kidney injury: an increasing global concern. Lancet 382:170–179

Lange C, Togel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, Westenfelder C (2005) Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. Kidney Int 68:1613–1617

Lasala GP, Silva JA, Minguell JJ (2012) Therapeutic angiogenesis in patients with severe limb ischemia by transplantation of a combination stem cell product. J Thorac Cardiovasc Surg 144:377–382

Lee EY, Xia Y, Kim WS, Kim MH, Kim TH, Kim KJ, Park BS, Sung JH (2009) Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. Wound Repair Regen 17:540–547

Lee MJ, Kim J, Kim MY, Bae YS, Ryu SH, Lee TG, Kim JH (2010) Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells. J Proteome Res 9:1754–1762

Lees JS, Sena ES, Egan KJ, Antonic A, Koblar SA, Howells DW, Macleod MR (2012) Stem cell-based therapy for experimental stroke: a systematic review and meta-analysis. Int J Stroke 7:582–588

Li H, Fu X, Ouyang Y, Cai C, Wang J, Sun T (2006) Adult bone-marrow-derived mesenchymal stem cells contribute to wound healing of skin appendages. Cell Tissue Res 326:725–736

Li T, Yan Y, Wang B, Qian H, Zhang X, Shen L, Wang M, Zhou Y, Zhu W, Li W, Xu W (2013) Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. Stem Cells Dev 22:845–854

Lim SY, Kim YS, Ahn Y, Jeong MH, Hong MH, Joo SY, Nam KI, Cho JG, Kang PM, Park JC (2006) The effects of mesenchymal stem cells transduced with Akt in a porcine myocardial infarction model. Cardiovasc Res 70:530–542

Linke A, Muller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, Castaldo C, Cascapera S, Bohm M, Quaini F et al (2005) Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci U S A 102:8966–8971

Little MH (2006) Regrow or repair: potential regenerative therapies for the kidney. J Am Soc Nephrol 17:2390–2401

Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY (2011) In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Sci Transl Med *3*:82ra39

Lopatina T, Kalinina N, Karagyaur M, Stambolsky D, Rubina K, Revischin A, Pavlova G, Parfyonova Y, Tkachuk V (2011) Adipose-derived stem cells stimulate regeneration of peripheral nerves: BDNF secreted by these cells promotes nerve healing and axon growth de novo. PLoS One 6:e17899

Lu P, Jones LL, Tuszynski MH (2005) BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Exp Neurol 191:344–360

Lu D, Chen B, Liang Z, Deng W, Jiang Y, Li S, Xu J, Wu Q, Zhang Z, Xie B, Chen S (2011) Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. Diabetes Res Clin Pract 92:26–36

Luan Y, Zhang X, Kong F, Cheng GH, Qi TG, Zhang ZH (2012) Mesenchymal stem cell prevention of vascular remodeling in high flow-induced pulmonary hypertension through a paracrine mechanism. Int Immunopharmacol 14:432–437

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H et al (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705

Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Fagioli F (2001) Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. Haematologica 86:1099–1100

Martin-Rendon E, Sweeney D, Lu F, Girdlestone J, Navarrete C, Watt SM (2008) 5-Azacytidine-treated human mesenchymal stem/progenitor cells derived from umbilical cord, cord blood and bone marrow do not generate cardiomyocytes in vitro at high frequencies. Vox Sang 95:137–148

Mathivanan S, Ji H, Simpson RJ (2010) Exosomes: extracellular organelles important in intercellular communication. J Proteome 73:1907–1920

McCoy MK, Martinez TN, Ruhn KA, Wrage PC, Keefer EW, Botterman BR, Tansey KE, Tansey MG (2008) Autologous transplants of Adipose-Derived Adult Stromal (ADAS) cells afford dopaminergic neuroprotection in a model of Parkinson's disease. Exp Neurol 210:14–29

Mcculloch EA, Till JE (1960) The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res 13:115–125

McFarlin K, Gao X, Liu YB, Dulchavsky DS, Kwon D, Arbab AS, Bansal M, Li Y, Chopp M, Dulchavsky SA, Gautam SC (2006) Bone marrow-derived mesenchymal stromal cells accelerate wound healing in the rat. Wound Repair Regen 14:471–478

Mirotsou M, Jayawardena TM, Schmeckpeper J, Gnecchi M, Dzau VJ (2011) Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. J Mol Cell Cardiol 50:280–289

Moon MH, Kim SY, Kim YJ, Kim SJ, Lee JB, Bae YC, Sung SM, Jung JS (2006) Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. Cell Physiol Biochem 17:279–290

Mora-Lee S, Sirerol-Piquer MS, Gutierrez-Perez M, Gomez-Pinedo U, Roobrouck VD, Lopez T, Casado-Nieto M, Abizanda G, Rabena MT, Verfaille C, Prosper F, Garcia-Verdugo JM (2012) Therapeutic effects of hMAPC and hMSC transplantation after stroke in mice. PLoS One 7: e43683

Morigi M, De Coppi P (2014) Cell therapy for kidney injury: different options and mechanisms–-mesenchymal and amniotic fluid stem cells. Nephron Exp Nephrol 126:59

Morigi M, Imberti B, Zoja C, Corna D, Tomasoni S, Abbate M, Rottoli D, Angioletti S, Benigni A, Perico N, Alison M, Remuzzi G (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

Morigi M, Introna M, Imberti B, Corna D, Abbate M, Rota C, Rottoli D, Benigni A, Perico N, Zoja C et al (2008) Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice. Stem Cells 26:2075–2082

Morigi M, Rota C, Montemurro T, Montelatici E, Lo Cicero V, Imberti B, Abbate M, Zoja C, Cassis P, Longaretti L et al (2010) Life-sparing effect of human cord blood-mesenchymal stem cells in experimental acute kidney injury. Stem Cells 28:513–522

Moriya K, Yoshikawa M, Saito K, Ouji Y, Nishiofuku M, Hayashi N, Ishizaka S, Fukui H (2007) Embryonic stem cells develop into hepatocytes after intrasplenic transplantation in CCl4-treated mice. World J Gastroenterol 13:866–873

Muller-Ehmsen J, Krausgrill B, Burst V, Schenk K, Neisen UC, Fries JW, Fleischmann BK, Hescheler J, Schwinger RH (2006) Effective engraftment but poor mid-term persistence of mononuclear and mesenchymal bone marrow cells in acute and chronic rat myocardial infarction. J Mol Cell Cardiol 41:876–884

Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ (2005) Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. Proc Natl Acad Sci U S A 102:18171–18176

Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura S (2004) Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 287:H2670–H2676

Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, Fujii T, Uematsu M, Ohgushi H, Yamagishi M et al (2005) Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 112:1128–1135

Neuhuber B, Timothy Himes B, Shumsky JS, Gallo G, Fischer I (2005) Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. Brain Res 1035:73–85

Nguyen BK, Maltais S, Perrault LP, Tanguay JF, Tardif JC, Stevens LM, Borie M, Harel F, Mansour S, Noiseux N (2010) Improved function and myocardial repair of infarcted heart by intracoronary injection of mesenchymal stem cell-derived growth factors. J Cardiovasc Transl Res 3:547–558

Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N (2007a) Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. FEBS Lett 581:3961–3966

Ohnishi S, Yasuda T, Kitamura S, Nagaya N (2007b) Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells 25:1166–1177

Ohnishi S, Yanagawa B, Tanaka K, Miyahara Y, Obata H, Kataoka M, Kodama M, Ishibashi-Ueda H, Kangawa K, Kitamura S, Nagaya N (2007c) Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J Mol Cell Cardiol 42(1):88–97

Ohta M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, Kataoka K, Chou H, Ishikawa N, Matsumoto N, Iwashita Y et al (2004) Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. Exp Neurol 187:266–278

Panepucci RA, Siufi JL, Silva WA Jr, Proto-Siquiera R, Neder L, Orellana M, Rocha V, Covas DT, Zago MA (2004) Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells 22:1263–1278

Parekkadan B, van Poll D, Megeed Z, Kobayashi N, Tilles AW, Berthiaume F, Yarmush ML (2007) Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. Biochem Biophys Res Commun 363:247–252

Pisano F, Altomare C, Cervio E, Barile L, Rocchetti M, Ciuffreda MC, Malpasso G, Copes F, Mura M, Danieli P et al (2015) Combination of miRNA499 and miRNA133 exerts a synergic effect on cardiac differentiation. Stem Cells 33:1187–1199

Pittenger MF, Martin BJ (2004) Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95:9–20

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

Pluchino S, Cossetti C (2013) How stem cells speak with host immune cells in inflammatory brain diseases. Glia 61:1379–1401

Pluchino S, Zanotti L, Brambilla E, Rovere-Querini P, Capobianco A, Alfaro-Cervello C, Salani G, Cossetti C, Borsellino G, Battistini L et al (2009) Immune regulatory neural stem/precursor cells protect from central nervous system autoimmunity by restraining dendritic cell function. PLoS One 4:e5959

Pollock K, Dahlenburg H, Nelson H, Fink KD, Cary W, Hendrix K, Annett G, Torrest A, Deng P, Gutierrez J et al (2016) Human mesenchymal stem cells genetically engineered to overexpress brain-derived neurotrophic factor improve outcomes in huntington's disease mouse models. Mol Ther 24:965–977

Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 109:1292–1298

Robey P (2017) "Mesenchymal stem cells": fact or fiction, and implications in their therapeutic use. F1000Research 6:1–8

Rodrigues M, Griffith LG, Wells A (2010) Growth factor regulation of proliferation and survival of multipotential stromal cells. Stem Cell Res Ther 1:32

Rota C, Imberti B, Pozzobon M, Piccoli M, De Coppi P, Atala A, Gagliardini E, Xinaris C, Benedetti V, Fabricio AS et al (2012) Human amniotic fluid stem cell preconditioning improves their regenerative potential. Stem Cells Dev 21:1911–1923

Roufosse CA, Direkze NC, Otto WR, Wright NA (2004) Circulating mesenchymal stem cells. Int J Biochem Cell Biol 36:585–597

Sadan O, Bahat-Stromza M, Barhum Y, Levy YS, Pisnevsky A, Peretz H, Ilan AB, Bulvik S, Shemesh N, Krepel D et al (2009) Protective effects of neurotrophic factor-secreting cells in a 6-OHDA rat model of Parkinson disease. Stem Cells Dev 18:1179–1190

Samura M, Hosoyama T, Takeuchi Y, Ueno K, Morikage N, Hamano K (2017) Therapeutic strategies for cell-based neovascularization in critical limb ischemia. J Transl Med 15(1):49. https://doi.org/10.1186/s12967-017-1153-4

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256

Sarojini H, Estrada R, Lu H, Dekova S, Lee MJ, Gray RD, Wang E (2008) PEDF from mouse mesenchymal stem cell secretome attracts fibroblasts. J Cell Biochem 104:1793–1802

Sasaki M, Abe R, Fujita Y, Ando S, Inokuma D, Shimizu H (2008) Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. J Immunol 180:2581–2587

Schiavetta A, Maione C, Botti C, Marino G, Lillo S, Garrone A, Lanza L, Pagliari S, Silvestroni A, Signoriello G, Sica V, Cobellis G (2012) A phase II trial of autologous transplantation of bone marrow stem cells for critical limb ischemia: results of the Naples and Pietra Ligure Evaluation of Stem Cells study. Stem Cells Transl Med 1:572–578

Schifferli JA (2011) Microvesicles are messengers. Semin Immunopathol 33:393–394

Sdrimas K, Kourembanas S (2014) MSC microvesicles for the treatment of lung disease: a new paradigm for cell-free therapy. Antioxid Redox Signal 21:1905–1915

Shintani A, Nakao N, Kakishita K, Itakura T (2007) Protection of dopamine neurons by bone marrow stromal cells. Brain Res 1186:48–55

Shu SN, Wei L, Wang JH, Zhan YT, Chen HS, Wang Y (2004) Hepatic differentiation capability of rat bone marrow-derived mesenchymal stem cells and hematopoietic stem cells. World J Gastroenterol 10:2818–2822

Siniscalco D, Giordano C, Galderisi U, Luongo L, Alessio N, Di Bernardo G, de Novellis V, Rossi F, Maione S (2010) Intra-brain microinjection of human mesenchymal stem cells decreases allodynia in neuropathic mice. Cell Mol Life Sci 67:655–669

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

Skalnikova H, Motlik J, Gadher SJ, Kovarova H (2011) Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. Proteomics 11:691–708

Smith AN, Willis E, Chan VT, Muffley LA, Isik FF, Gibran NS, Hocking AM (2010) Mesenchymal stem cells induce dermal fibroblast responses to injury. Exp Cell Res 316:48–54

Snykers S, Vanhaecke T, Papeleu P, Luttun A, Jiang Y, Vander Heyden Y, Verfaillie C, Rogiers V (2006) Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. Toxicol Sci 94:330–341. discussion 235-9

Song H, Cha MJ, Song BW, Kim IK, Chang W, Lim S, Choi EJ, Ham O, Lee SY, Chung N, Jang Y, Hwang KC (2010) Reactive oxygen species inhibit adhesion of mesenchymal stem cells implanted into ischemic myocardium via interference of focal adhesion complex. Stem Cells 28:555–563

Sun X, Meng H, Wan W, Xie M, Wen C (2019) Application potential of stem/progenitor cell-derived extracellular vesicles in renal diseases. Stem Cell Res Ther 10:8

Takahashi M, Li TS, Suzuki R, Kobayashi T, Ito H, Ikeda Y, Matsuzaki M, Hamano K (2006) Cytokines produced by bone marrow cells can contribute to functional improvement of the

infarcted heart by protecting cardiomyocytes from ischemic injury. Am J Physiol Heart Circ Physiol 291:H886–H893
Tan CY, Lai RC, Wong W, Dan YY, Lim SK, Ho HK (2014) Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. Stem Cell Res Ther 5:76
Terrovitis JV, Smith RR, Marban E (2010) Assessment and optimization of cell engraftment after transplantation into the heart. Circ Res 106:479–494
Thery C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9:581–593
Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222
Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM, Choo A, Lee CN, Pasterkamp G, de Kleijn DP (2007) Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Res 1:129–137
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 Ren Physiol 289:F31–F42
Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C (2007) Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am J Physiol Ren Physiol 292:F1626–F1635
Togel F, Zhang P, Hu Z, Westenfelder C (2009) VEGF is a mediator of the renoprotective effects of multipotent marrow stromal cells in acute kidney injury. J Cell Mol Med 13: 2109–2114
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
Tomasoni S, Longaretti L, Rota C, Morigi M, Conti S, Gotti E, Capelli C, Introna M, Remuzzi G, Benigni A (2013) Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. Stem Cells Dev 22:772–780
Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, Jia ZQ (1999) Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 100:II247–II256
Tomita S, Mickle DA, Weisel RD, Jia ZQ, Tumiati LC, Allidina Y, Liu P, Li RK (2002) Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. J Thorac Cardiovasc Surg 123:1132–1140
Tondreau T, Dejeneffe M, Meuleman N, Stamatopoulos B, Delforge A, Martiat P, Bron D, Lagneaux L (2008) Gene expression pattern of functional neuronal cells derived from human bone marrow mesenchymal stromal cells. BMC Genomics 9:166-2164-9-166
Uccelli A, Pistoia V, Moretta L (2007) Mesenchymal stem cells: a new strategy for immunosuppression? Trends Immunol 28:219–226
Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736
Uccelli A, Benvenuto F, Laroni A, Giunti D (2011) Neuroprotective features of mesenchymal stem cells. Best Pract Res Clin Haematol 24:59–64
Uemura R, Xu M, Ahmad N, Ashraf M (2006) Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res 98:1414–1421
van Poll D, Parekkadan B, Cho CH, Berthiaume F, Nahmias Y, Tilles AW, Yarmush ML (2008) Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. Hepatology 47:1634–1643
Wakabayashi K, Nagai A, Sheikh AM, Shiota Y, Narantuya D, Watanabe T, Masuda J, Kobayashi S, Kim SU, Yamaguchi S (2010) Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. J Neurosci Res 88:1017–1025
Wakitani S, Saito T, Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417–1426

Wei X, Du Z, Zhao L, Feng D, Wei G, He Y, Tan J, Lee WH, Hampel H, Dodel R et al (2009) IFATS collection: the conditioned media of adipose stromal cells protect against hypoxia-ischemia-induced brain damage in neonatal rats. Stem Cells 27:478–488

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370

Wu Y, Chen L, Scott PG, Tredget EE (2007) Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells 25:2648–2659

Xin H, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M (2013) Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. J Cereb Blood Flow Metab 33:1711–1715

Xu X, Xu Z, Xu Y, Cui G (2005) Effects of mesenchymal stem cell transplantation on extracellular matrix after myocardial infarction in rats. Coron Artery Dis 16:245–255

Xu M, Uemura R, Dai Y, Wang Y, Pasha Z, Ashraf M (2007) In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function. J Mol Cell Cardiol 42:441–448

Yamamoto H, Quinn G, Asari A, Yamanokuchi H, Teratani T, Terada M, Ochiya T (2003) Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. Hepatology 37:983–993

Yu B, Gong M, Wang Y, Millard RW, Pasha Z, Yang Y, Ashraf M, Xu M (2013) Cardiomyocyte protection by GATA-4 gene engineered mesenchymal stem cells is partially mediated by translocation of miR-221 in microvesicles. PLoS One 8:e73304

Yulyana Y, Ho IA, Sia KC, Newman JP, Toh XY, Endaya BB, Chan JK, Gnecchi M, Huynh H, Chung AY, Lim KH, Leong HS, Iyer NG, Hui KM, Lam PY (2015) Paracrine factors of human fetal MSCs inhibit liver cancer growth through reduced activation of IGF-1R/PI3K/Akt signaling. Mol Ther 23:746–756

Zhang HC, Liu XB, Huang S, Bi XY, Wang HX, Xie LX, Wang YQ, Cao XF, Lv J, Xiao FJ, Yang Y, Guo ZK (2012) Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both in vitro and in vivo. Stem Cells Dev 21:3289–3297

Zhang Y, Chopp M, Meng Y, Katakowski M, Xin H, Mahmood A, Xiong Y (2015) Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. J Neurosurg 122:856–867

Zhao W, Li JJ, Cao DY, Li X, Zhang LY, He Y, Yue SQ, Wang DS, Dou KF (2012) Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis. World J Gastroenterol 18:1048–1058

Zuccato C, Marullo M, Vitali B, Tarditi A, Mariotti C, Valenza M, Lahiri N, Wild EJ, Sassone J, Ciammola A et al (2011) Brain-derived neurotrophic factor in patients with Huntington's disease. PLoS One 6:e22966

Peripheral Blood Mononuclear Cell Secretome for Tissue Repair

Lucian Beer, Elisabeth Simader, Michael Mildner, Mariann Gyöngyösi, and Hendrik Jan Ankersmit

Contents

Modified version of Beer L., Mildner, M., Gyöngyösi, M. et al. (2016) Peripheral blood mononuclear cell secretome for tissue repair. Apoptosis, Springer US

L. Beer
Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna, Austria

Cristian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Vienna, Austria
e-mail: lucian.beer@meduniwien.ac.at

E. Simader
Cristian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Vienna, Austria

Department of Thoracic Surgery, Medical University Vienna, Vienna, Austria

M. Mildner
Department of Dermatology, Medical University of Vienna, Vienna, Austria
e-mail: michael.mildner@meduniwien.ac.at

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_61

Abstract

For almost two decades, cell-based therapies have been tested in modern regenerative medicine to either replace or regenerate human cells, tissues, or organs and restore normal function. Secreted paracrine factors are increasingly accepted to exert beneficial biological effects that promote tissue regeneration. These factors are called the cell secretome and include a variety of proteins, lipids, microRNAs, and extracellular vesicles, such as exosomes and microparticles. The stem cell secretome has most commonly been investigated in preclinical settings. However, a growing body of evidence indicates that other cell types, such as peripheral blood mononuclear cells (PBMCs), are capable of releasing significant amounts of biologically active paracrine factors that exert beneficial regenerative effects. The apoptotic PBMC secretome has been successfully used preclinically for the treatment of acute myocardial infarction, chronic heart failure, spinal cord injury, stroke, and wound healing. In this review we describe the benefits of choosing PBMCs instead of stem cells in regenerative medicine and characterize the factors released from apoptotic PBMCs. We also discuss preclinical studies with apoptotic cell-based therapies and regulatory issues that have to be considered when conducting clinical trials using cell secretome-based products. This should allow the reader to envision PBMC secretome-based therapies as alternatives to all other forms of cell-based therapies.

1 Introduction

The concept of using paracrine factors in a therapeutic context in regenerative medicine can be seen as a consequence of the stem cell theory in the beginning of the twenty-first century. Here we will discuss the beginnings of stem cell research, with its promising initial results and subsequent disillusion, followed by the development of the secretome concept in regenerative medicine. We will discuss the term "secretome," characterize the components of the cell secretome, describe its effects in preclinical and clinical studies, and then outline the challenges and opportunities of secretome-based therapies in regenerative medicine.

M. Gyöngyösi
Division of Cardiology, Medical University of Vienna, Vienna, Austria
e-mail: mariann.gyongyosi@meduniwien.ac.at

H. J. Ankersmit (✉)
Cristian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Vienna, Austria

Head FFG Project 852748 "APOSEC", Vienna, Austria

Department of Thoracic Surgery, Medical University Vienna, Vienna, Austria
e-mail: hendrik.ankersmit@meduniwien.ac.at

2 Development of the Paracrine/Secretome Hypothesis

The origin of modern cell-free therapies can be traced back to the late nineteenth and early twentieth centuries. The Swiss physician Paul Niehans (1882–1971) coined the term "cell therapy." His cell therapy patients included celebrities such as Winston Churchill, Pope Pius XII, Agha Kahn, and Charles de Gaulle. Niehans extensively evaluated the effects of endogene rejuvenation via injection of xenogeneic cell suspensions derived from the endocrine glands, heart, kidney, liver, bone marrow, intestinal mucosa, and reticuloendothelial system into the corresponding organ, studying whether normal function was restored. He also studied blood enriched with leukocytes and hypothesized that this form of treatment could be used for a myriad of degenerative diseases and cancer. Niehans used either fresh xenogeneic cells in suspension or lyophilized xenogeneic cells (vacuum freeze-dried) derived from young animals, such as sheep or animal fetuses, or human fetal cells (Niehans 1952). Based on his experience with 3000 treated patients, Niehans postulated that xenogeneic cell infusion was well tolerated and not associated with any adverse reaction. Interestingly, as early as 1955, Rietschel and Pischinger articulated their reservations regarding the implantation of xenogeneic cells based on their data that xenograft transplantation induces a xenogeneic immune response, as well as the risk of transmitting zoonosis (Rietschel 1954).

Prior to Niehans, physicians experimented with stem cell-like cell types, including Alexander Maximow in 1909 during his work on blood formation (Maximow 1909). In addition, Elie Metchnikoff, and later Alexander Bogomolets, worked on the development of an adjuvant immunotherapy consisting of cytotoxic sera thought to prolong life and attenuate chronic diseases. (Metchnikoff 2004).

However, even though these researchers were enthusiastic proponents of their own theories and treatment options, the efficacy has not been demonstrated in a systematic manner. By modern standards, their results never progressed beyond an expert opinion and the publication of case reports. The lack of well-conducted studies in the field of "cell therapies" was the cause of well-argued skepticism throughout the twentieth century.

The current concept of using autologous or allogeneic stem cells for replacing or engineering injured tissues to reestablish normal biological function was re-envisioned in the beginning of the twenty-first century in the clinical setting of acute myocardial infarction (AMI) and chronic heart failure (HF). Initially, it was thought that the human heart is a postmitotic and terminally developed organ with no cell renewal capacity, but this dogma was refuted in 2001 by showing enhanced myocyte cell proliferation following AMI (Beltrami et al. 2001), which was confirmed by other groups (Bergmann et al. 2009, 2015). Further studies revealed that a small amount of extra-cardiac progenitor cells is capable of migrating into the damaged human myocardium following heart transplantation to replace damaged cardiomyocytes (Laflamme et al. 2002). A variety of preclinical studies simultaneously showed striking results by using different sources of stem cells for the treatment of AMI. Human bone marrow-derived CD34 cells attenuated myocardial infarction in athymic rats (impaired immune system) when injected intravenously

48 h after vascular ligation (Kocher et al. 2001). Orlic et al. showed that intramyocardial injection of murine c-KIT+ bone marrow-derived cells in mice improves hemodynamic functions; the authors claimed that these stem cells enhanced myocardial regeneration by de novo generation of functional myocardium (Orlic et al. 2001).

Based on these encouraging preclinical experiments, several groups rapidly transferred the "stem cell potential" to the realm of clinical application. Initial trials, such as the randomized controlled trial (RCT) TOPCARE-AMI, were able to show minor improvements in left ventricular function following intracoronary injection of autologous progenitor cells (Assmus et al. 2002). However, ambiguous results were published in subsequent years without providing evidence that a single-cell-based therapy has significant effectiveness (Behfar et al. 2014). However, three recent meta-analyses generated contradictory results. Fisher et al. reported that autologous cell therapy may reduce the risk of mortality and re-hospitalization in patients with HF (Fisher et al. 2015, 2016). Gyöngyösi et al. showed that intracoronary stem cell therapy following AMI provides no clinical benefits or reduction in mortality (Gyöngyösi et al. 2015). Gyöngyösi et al. performed an individual participant data (IPD)-based meta-analysis consisting of 12 RCTs with 1252 patients and concluded that neither clinical data nor left ventricular functions were significantly improved by intracoronary cell therapy following AMI (Gyöngyösi et al. 2015). These data highlight the lack of clinical efficacy of stem cell therapy in the clinical setting of AMI and dampen the enthusiasm for resource-intensive research into cell-based therapy. The controversial discussion on the efficacy of stem cell therapy is ongoing, and the current BAMI trial funded by the European Commission (ClinicalTrials.gov Identifier: NCT01569178) will provide definitive answers in a well-designed and controlled study.

How can we interpret the data generated during the last two decades in stem cell research, and, more importantly, what lessons have been learned from these investigations? Given that the injection of diverse types of stem cells can promote cardiomyocyte cytoprotection/regeneration preclinically, it was initially thought that these stem cells incorporate into the injured organ and transdifferentiate into cardiomyocytes (Orlic et al. 2001). Later, it was shown that mesenchymal stem cells (MSCs) not only engraft and differentiate into cardiomyocytes but also stimulate the proliferation and differentiation of endogenous cardiac stem cells (Hatzistergos et al. 2010), and allogeneic MSCs stimulate the proliferation of endothelial progenitor cells, restoring normal endothelial function (Premer et al. 2015).

In contrast, only a small percentage of injected stem cells engraft in the heart (Hodgkinson et al. 2016). Penicka et al. reported that approximately 5% and 1% of stem cells remain in the human myocardium 2 and 18 h after intracoronary infusion, respectively (Losordo et al. 2007; Mansour et al. 2010). The vast majority of cells were sequestered in the spleen, liver, lung, bone marrow, and lymph nodes a few hours after infusion (Chen et al. 2004; Mansour et al. 2010). Subsequent studies could not reproduce the stem cell differentiation into cardiac cells. Specifically, c-KIT+ cardiac progenitor cells (Balsam et al. 2004) were unable to differentiate

into cardiomyocytes or support myocardial regeneration following myocardial infarction. Murry et al. failed to identify any transdifferentiation of hematopoietic stem cells into cardiomyocytes in 145 transplants into normal and injured adult mouse hearts (Murry et al. 2004).

If stem cells do not engraft in a biologically relevant manner or demonstrate long-term survival in injured heart, how can we explain the beneficial effects (Nguyen et al. 2011)? Recent evidence suggests that, instead of direct cell fusion or transdifferentiation, paracrine factors released by injected cells are responsible for the beneficial effects. Gnecchi, using in vivo data, was one of the first to propose that infused stem cells may exert their biological effects via the secretion of paracrine factors in addition to cell-cell interactions. In 2005, this group reported that conditioned medium from MSCs under hypoxic stress significantly attenuated hypoxia-induced cell death among adult rat ventricular cardiomyocytes (Kwak and Mach 2005). Gnecchi and Dzau demonstrated the importance of paracrine factors derived from stem cells in tissue regeneration in several studies and reviews (Gnecchi et al. 2008, 2016; Gnecchi and Melo 2009; Mirotsou et al. 2007). Another pioneer in the field is Prof. Dr. KC Wollert, who speculated in 2005 about the role of paracrine factors from stem cells in regenerative medicine (Wollert and Drexler 2005). Interestingly, in 2008 the same group showed that supernatants obtained from PBMCs have only marginal differences from the stem cell secretome regarding the ability to promote cell proliferation. In addition, ProteinChip and GeneChip analysis of paracrine factors revealed minor differences (Korf-Klingebiel et al. 2008), supporting the study of alternative mechanisms underlying nucleated stem cell therapy in the field of regenerative medicine.

3 Death of the Stem Cell Hypothesis

Cell necrosis-induced inflammatory reactions are an integral part of the pathophysiology of myocardial ischemia. These inflammatory processes augment cardiomyocyte dysfunction and ventricular remodeling. In 2005, Thum et al. proposed that immunomodulatory signals released by injected apoptotic stem cells may be responsible for the beneficial effects observed in the initial clinical trials utilizing ex vivo manipulated stem cells (Thum et al. 2005). They drew attention to the fact that 5–25% of all injected stem cells are apoptotic (Wollert et al. 2004) and proposed that these dying cells attenuate inflammatory reactions via the induction of anti-inflammatory cytokines (e.g., TGF-β, IL-10) by macrophages. This hypothesis was supported by the observation that apoptotic neutrophils augment TGF-β, prostaglandin E2 (PGE2), and platelet activation factor expression in phagocytes, whereas inflammatory mediators, such as IL-1β, IL-8, and TNF-α, were inhibited (Fadok et al. 1998). However, Thum et al. not only hypothesized that beneficial local immune reactions are induced by apoptotic stem cells but also speculated that any other nucleated cell type undergoing programmed cell death may improve myocardial regeneration.

4 Proof and Extension of Thum's Apoptosis Hypothesis

Thum's hypothesis was proven by Ankersmit et al., who sought to answer two major questions using a wild-type rodent model of AMI: Are PBMCs capable of attenuating myocardial damage when injected intravenously after coronary ligation, and does the induction of apoptosis in PBMCs prior to injection alter their biological activity?

Using in vitro assays, Ankersmit et al. were able to show that gamma-irradiated apoptotic PBMCs attenuated lipopolysaccharide (LPS)-induced spillage of IL-6 and IL-1β in co-cultured monocytes and PBMCs (Ankersmit et al. 2009). In addition, irradiated PBMCs reduced T-cell proliferation in mixed lymphocyte reaction assays (Ankersmit et al. 2009).

In in vivo experiments using a rodent model of AMI induced by ligation of the left anterior descending (LAD) artery, the infusion of irradiated PBMC suspensions (apoptotic PBMC-S) reduced infarct size compared to animals receiving non-irradiated PBMCs (living PBMC-S) or cell culture medium alone. Functional parameters (ejection fraction, end-systolic and end-diastolic diameters) were improved in animals treated with apoptotic PBMC-S (Ankersmit et al. 2009). In line with these findings, qualitative and quantitative changes were observed in myocardial cell infiltrates 72 h after AMI in rats receiving apoptotic PBMC-S. In contrast, rats receiving non-irradiated PBMCs or control media had a mixed cellular infiltrate in the border zone (e.g., fibroblasts, neutrophils, lymphomononuclear cells, macrophages/monocytes, endothelial cells, and dystrophic cardiomyocytes), and rats treated with irradiated PBMCs had a massive infiltration of CD68+ mononuclear cells (monocytes/macrophages). These animals also had cells positive for c-KIT+ (CD117) and vascular endothelial growth factor receptor 2 (VEGF-R2) (Ankersmit et al. 2009). Ankersmit et al. also tested whether conditioned medium from apoptotic PMBCs may influence gene expression by fibroblasts and keratinocytes in vitro. Surprisingly, the co-incubation of human primary fibroblasts and keratinocytes with paracrine factors derived from apoptotic PBMCs induced a massive upregulation of MMP-9, VEGF, and IL-8. Thus, this study showed that apoptotic PBMC secretome exerts anti-inflammatory effects and reduces myocardial injury following AMI and that the supernatants from these cells induce the expression of pro-angiogenic genes in vitro. Looking at the condition media data, it was obvious to us that the secretome of nucleated apoptotic PBMCs is the only explanation and easily obtainable solution in regenerative medicine.

5 Preclinical Application of Nucleated Apoptotic Cells

Based on the immunomodulatory capacities of apoptotic cells during the last decade, several preclinical studies evaluated their usage for the treatment of autoimmune disease or organ transplantation (reviewed in Saas et al. 2016).

Gray et al. reported that the infusion of apoptotic thymocytes attenuated the severity or prevented the development of collagen-induced arthritis in mice via

the modulation of regulatory B cells and CD4+ T-cells (Gray et al. 2007). Perruche et al. published comparable results using streptococcal cell wall-induced arthritis in rats. The intraperitoneal injection of gamma-irradiated apoptotic thymocytes at the time of immunization reduced disease severity (Perruche et al. 2009). In a murine model of methylated BSA-induced arthritis, the application of etoposide induced apoptotic dendritic cells, but LPS-activated apoptotic dendritic cells inhibited arthritis (Notley et al. 2015). In addition, Grau et al. used an autoimmune-mediated colitis model to evaluate the effect of gamma-irradiated apoptotic splenocytes or human apoptotic mononuclear cells. The authors were able to show that apoptotic cells attenuated pro-inflammatory cytokine release from macrophages and the severity of colitis (Grau et al. 2015).

Holzinger et al. harvested autologous PBMCs from diabetic patients with venous foot ulcers and stimulated them ex vivo with phytohemagglutinin. They then applied these cell suspensions to the foot ulcers. The clinical effect was a significant enhancement of granulation and epithelialization of the skin ulcers (Holzinger et al. 1994).

In an unrecognized citation, in 1970 Földes et al. investigated whether the injection of antilymphocyte serum, which induces apoptosis in PBMCs in vivo and vitro (Ankersmit et al. 2002), is able to attenuate experimental AMI (Földes et al. 1970). In their historic work, they were able to show that the injection of antilymphocyte serum immediately decreased ischemic myocardial damage and arrhythmia in experimental AMI. They attributed these effects to the immunosuppressive effects of the antilymphocyte serum. This therapy concept was confirmed and extended by Lichtenauer et al. in 2012. Lichtenauer et al. injected the commercially available immunosuppressive agent rabbit ATG (rATG, Thymoglobulin, Genzyme, Germany) into rodents exposed to permanent LAD ligation (Lichtenauer et al. 2012). rATG is a successfully applied drug in clinical transplant immunology that has a mechanism comparable to antilymphocyte serum. Experimental in vivo ATG treatment reduced the area of necrosis and improved myocardial function compared to control treatment. In vitro data confirmed that ATG induced the release of several pro-angiogenic proteins from rat and human PBMCs into the supernatant, such as CXLC8 (IL-8). Furthermore, these paracrine factors induced the down-regulation of p53 in cultured human cardiomyocytes, suggesting a direct cytoprotective effect. The authors concluded that, in vivo, ATG induces the apoptosis of lymphocytes, which release paracrine factors that reduce ventricular remodeling and improve cardiac function after experimental AMI (Lichtenauer et al. 2012).

Thus, most of these studies injected apoptotic splenocytes or thymocytes, and only two studies induced leukocyte apoptosis in vivo. The proposed mechanisms for these cell-based therapies include immunomodulatory effects induced by the clearance of apoptotic cells by phagocytes and characterized by the direct release of immunosuppressive factors (e.g., IL-10, TGF-β) (Saas et al. 2016). Whether the injection of nucleated cells or their released factors is sufficient to induce the immunomodulatory systemic reactions has not yet been studied.

6 Paradigm Changes in Tissue Repair: Validation of the PBMC Secretome

Initially, Ankersmit's group was able to show that the injection of paracrine factors released from apoptotic PBMCs attenuates myocardial damage and preserves myocardial function following AMI in a rodent model and clinically more relevant large animal model (Lichtenauer et al. 2011b). Pigs receiving apoptotic cell secretome had an increased ejection fraction (57.0 vs. 40.5%), improved cardiac output (4.0 vs. 2.4 l/min), and reduced infarct area (12.6 vs. 6.9%) compared to controls. Lichtenauer et al. showed that cardiomyocytes exposed to apoptotic PBMC-S increased pro-survival proteins (AKT, ERK1/2, CREB, c-Jun) and anti-apoptotic genes (Bcl-2, BAG1) and prevented starvation-induced cell death in primary cultured human cardiomyocytes. These in vitro data indicated that apoptotic PBMC-S enhances endogenous myocardial protection and repair following AMI (Lichtenauer et al. 2011b).

Hoetzenecker et al. showed that, in addition to an effect on intracellular myocardial signaling cascades, apoptotic PBMC-S preserved porcine epi/myocardial microvascular perfusion in a closed chest reperfusion infarction model (Hoetzenecker et al. 2012). Microvascular obstruction was measured by functional magnetic resonance imaging (fMRI) and cardiac catheterization in treated animals. Both methods revealed improved microvascular blood flow in treated animals. In addition, apoptotic PBMC-S inhibited platelet activation via the upregulation of eNOS, iNOS, and vasodilator-stimulated phosphoprotein (VASP) activation in cultured HUVECs in vitro, indicating that apoptotic PBMC-S elicited NO-dependent systemic vasodilation (Hoetzenecker et al. 2012). These data indicate that apoptotic PBMC-S not only caused direct cytoprotective effects in injured hearts but also induced systemic effects on circulating platelets and endothelial cells.

This work was extended by Pavo et al., who were able to demonstrate that apoptotic PBMC-S exerts regenerative effects in a latent porcine chronic post-myocardial infarction model (Pavo et al. 2014). In these experiments, pigs were subjected to a 90-min occlusion followed by reperfusion of the mid-left ventricular descending coronary artery. Thirty days later, the animals received either apoptotic PBMC-S or control medium in the border zone after myocardial infarction via a NOGA catheter. On day 60, they were able to show that secretome-treated animals had a significantly reduced infarct size and improved cardiac index compared to controls. Transcriptional gene analysis of the myocardium revealed higher expression of protective myogenic factor MEFC2 and reduced expression of pro-apoptotic genes in treated pigs (Pavo et al. 2014). These data led the authors to conclude that apoptotic PBMC-S promotes curative gene signatures as late as 30 days after the application of PBMC-S in animals with chronic ischemic left ventricular dysfunction.

The pathophysiology and molecular processes following ischemic stroke are somewhat similar to those of AMI. Following vascular obstruction due to a ruptured atherosclerotic plaque or arterial thrombus, the downstream cells become hypoxic and eventually apoptotic/necrotic. As in myocardial infarction, immediate

restoration of blood flow is the first-line treatment in patients with acute ischemic stroke. Altmann et al. showed that, in a rodent model of cerebral stroke, the administration of apoptotic cell secretome reduced the ischemic area by 36% and improved the functional neurological outcome (Altmann et al. 2014). In vitro assays revealed enhanced sprouting of human neurons and the upregulation of pro-survival proteins (CREP, ERK1/2, c-Jun, Hsp27) in primary cultured astrocytes and Schwann cells. Interestingly, the intraperitoneal application of human apoptotic PBMC-S in naïve rodents massively increased the secretion of rat brain-derived neurotrophic factor (BDNF), a neuroprotective peptide known to be involved in neurogenesis. These data further support apoptotic PBMC-S having not only direct regenerative/cytoprotective effects on injured organs but also systemic effects, overcoming tissue damage and cell death in the acute phase of hypoxia.

Similar to stroke, the neurological damage following acute spinal cord injury initiates a short initial trauma followed by a second injury caused by the endogenous immune response to the traumatic injury. This second undesirable phase of spinal cord injury consists of a multilayered and complex combination of inflammatory pathways that are thought to drive ongoing tissue damage via enzymatic degradation, oxidative stress, and blood-brain barrier dysfunction (David et al. 2012). Haider et al. investigated the role of the apoptotic cell secretome in a rodent spinal cord injury model (Haider et al. 2015). Rats treated with paracrine factors from irradiated PBMCs had significantly improved neurological function compared to the control group. Histological evaluation of spine sections revealed a smaller spinal cord cavity, reduced axonal damage, and improved vascularity index around the lesion. The PBMC secretome enhanced angiogenesis in aortic ring assays and spinal cord tissue. The authors also tested whether an immunological mechanism may be involved in neuroprotection. The secretome-treated animals exhibited enhanced recruitment of CD68-positive cells with parallel reduction in the levels of iNOS 3 days after injury. These data indicate that the PBMC secretome increases the presence of cells with known beneficial anti-inflammatory effects after spinal cord injury that accelerate the clearance of immunological disturbances, attenuating the secondary damage after spinal cord injury (Haider et al. 2015).

The PBMC secretome has been shown to enhance wound healing in vivo and in vitro (Hacker et al. 2016; Mildner et al. 2013). Mice with artificial dermal wounds were treated with emulsion containing either PBMC secretome or control medium. Six days after injury, wound closure was significantly enhanced in secretome-treated animals compared to control animals. Histological evaluation showed accelerated reepithelialization and neo-angiogenesis. In line with these in vivo data, experiments with keratinocytes and fibroblasts showed enhanced proliferation and upregulation of pro-survival signaling pathways after co-incubation with PBMC secretome (Mildner et al. 2013). In a clinically relevant pig skin burn model, Hacker et al. reported that PBMC secretome accelerated wound quality, augmented neo-angiogenesis, and decreased the number of mast cells (Hacker et al. 2016).

In recently published work, Hoetzenecker et al. investigated the capacity of apoptotic PBMC-S to modulate myocardial inflammation in a CD4-positive T-cell-dependent murine model of autoimmune myocarditis. Mice receiving apoptotic

PBMC-S at the clinical onset of disease had reduced lymphocytic infiltrate, offering a novel treatment option in an experimental myocarditis model. The authors showed that PBMC-S induced caspse-8-dependent apoptosis of autoreactive CD-4-positive T-cells, leading to remission of the decorative autoimmune processes (Hoetzenecker et al. 2015) in a well-defined myocarditis model.

The immunomodulatory effects of PBMC-S were further evaluated by Kasiri et al. (2016). In this study the authors were able to show that PBMC-S contains significant amounts of antimicrobial peptides. In vitro experiments revealed antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria. In addition, intravenous application of apoptotic PBMC-S in rats increased de novo endogenous AMP production in vivo. The authors deduced that PBMC-S has direct and indirect positive effects on the immune system.

Based on these data, we concluded that apoptotic PBMC-S initiates multiple modes of action (MOAs) that eventually lead to cytoprotection and prevention of hypoxic tissue in vivo (Table 1). The production process of these paracrine factors is depicted in Fig. 1.

7 The Quest for the Biologically Active Component(s) of Apoptotic PBMC Secretome

A plethora of unresolved questions are still unanswered regarding which component (s), concentration(s), and mode(s) of delivery are responsible for the effects of the PBMC secretome. A single molecule may regulate a broad spectrum of different processes, including platelet inhibition, vasodilation, neo-angiogenesis, and cytoprotection. Another option is a pleiotropy of factors alone or in combination that orchestrate different molecular cascades, leading to the beneficial effects (Fig. 2). In order to determine the MOA of the PBMC secretome, we first have to define the secretome itself.

Using a systemic approach, Beer et al. tried to classify the secretome components of gamma-irradiated apoptotic PBMCs (Beer et al. 2014) based on their principle molecular characteristics in proteins (e.g., cytokines and growth factors), extracellular vesicles (e.g., microparticles and exosomes), and lipids. Lichtenauer et al. performed cytokine membrane arrays using supernatant from irradiated and non-irradiated PBMCs; particularly when stressed, the supernatants contained high amounts of chemokines (e.g., CXCL8, CXCL5, CXCL1, CCL5, VEGF) (Lichtenauer et al. 2011b). We also utilized a bioinformatics-based approach in in vitro cultured PBMCs and irradiated PBMCs. A total of 213 putative actively secreted proteins were identified in the transcriptome of irradiated PBMCs and 167 transcripts in non-irradiated PBMCs (Beer et al. 2015), highlighting that irradiation causes PBMCs to become a highly active "bioreactor" while undergoing programmed cell death.

A growing body of evidence indicates that lipids released from apoptotic cells exert paracrine biological activity (Boland et al. 2013; Lauber et al. 2003). Using the PBMC secretome, we focused on oxidized phospholipids because this group of

Table 1 Preclinical and clinical application of apoptotic PBMC-S

Species	Experimental model	Effects on disease	Application	Concentration at cultivation	PBMC source	Apoptotic stimulus	References
Rat	AMI	Reduced infarct size, improved functional parameters	i.v.	25×10^6	Syngen	γ-irradiation (60 Gy)	Lichtenauer et al. (2011a)
Pig	AMI		i.v.	25×10^6	Syngen	γ-irradiation (60 Gy)	Lichtenauer et al. (2011b)
Pig	AMI		i.v.	25×10^6	Syngen	γ-irradiation (60 Gy)	Hoetzenecker et al. (2012)
Mice	EAM	Resolution of acute inflammation	i.p.	25×10^6	Syngen	γ-irradiation (60 Gy)	Hoetzenecker et al. (2015)
Mice	Dermal wound	Improved wound healing	Topical	25×10^6	Syngen	γ-irradiation (60 Gy)	Mildner et al. (2013)
Pig	Chronic HF	Improved functional parameters	i.m.	25×10^6	Syngen	γ-irradiation (60 Gy)	Pavo et al. (2014)
Rat	Stroke	Reduced infarct size, improved neurological parameters	i.v.	25×10^6	Syngen/human GMP viral cleared	γ-irradiation (60 Gy)	Altmann et al. (2014)
Rat	SCI	Reduced trauma size, improved neurological parameters	i.p.	25×10^6	Human GMP viral cleared	γ-irradiation (60 Gy)	Haider et al. (2015)
Pig	Dermal wound	Improved wound healing	Topical	25×10^6	Human	γ-irradiation (60 Gy)	Hacker et al. (2016)
Pig	AMI	Reduced infarct size, improved functional parameters	i.v.	25×10^6	Syngen GMP viral cleared	γ-irradiation (60 Gy)	Beer et al. (2015)
Human	Dermal wound	Safety and tolerability	Topical	25×10^6	Autologous GMP	γ-irradiation (60 Gy)	ClinicalTrials.gov identifier: NCT02284360[a]

AMI acute myocardial infarction, *EAM* experimental autoimmune myocarditis, *HF* heart failure, *SCI* spinal cord injury, *i.v.* intravenous, *i.p.* intraperitoneal, *i.m.* intramuscular

[a]Study report – synopsis: http://www.aposcience.at/fileadmin/user_upload/MarsyasI-Clinical_study-Study_report_Synopsis.pdf

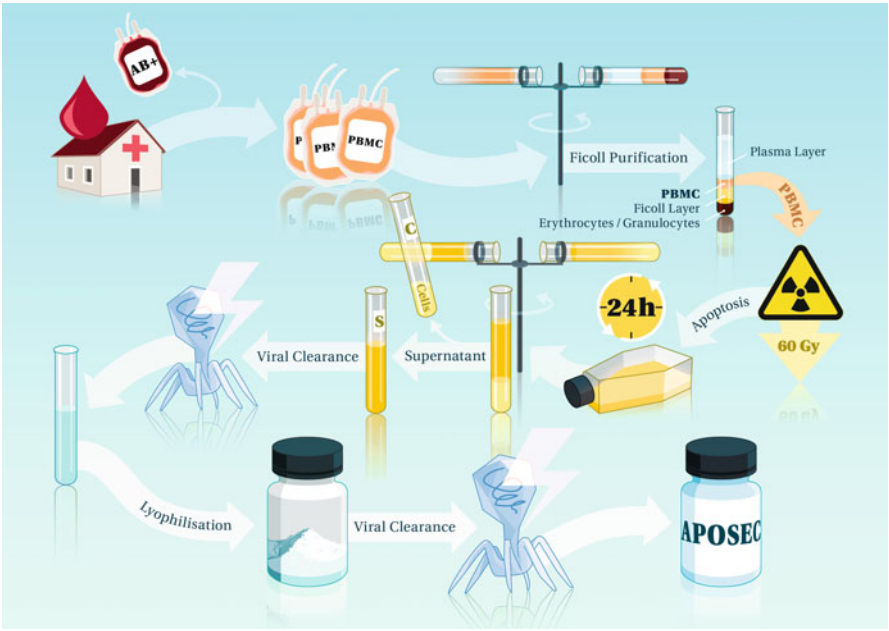


Fig. 1 Schematic workflow of the preparation of apoptotic PBMC secretome. PBMC-enriched blood bags are obtained by blood banks and used for the generation of paracrine factors. The PBMCs are further purified using Ficoll-Paque centrifugation. Apoptosis is induced by 60 Gy gamma irradiation. The PBMCs are then cultivated at 37 °C in 5% CO/5% CO2 for 24 h. The cells are removed by centrifugation and ultrafiltration. The remaining cell culture supernatant containing the paracrine factors is subjected to methylene blue viral clearance. The viral cleared supernatant is then lyophilized to remove all soluble factors. The remaining solid compartments are subjected to a second viral clearance step and irradiated with 30 k Gy, eventually yielding the final product produced in accordance with GMP

lipids has pleiotropic biological effects ranging from the induction of endothelial cell proliferation and angiogenesis to the attenuation of Toll-like receptor (TLR)-induced inflammation and the inhibition of oxidative burst in neutrophil granulocytes. Using high-pressure lipid chromatography, we measured increased concentrations of oxidized phospholipids in supernatant from irradiated PBMCs, especially PLPC-OOH, PAPC-OOH, SGPC, and PGPC (Beer et al. 2015).

In addition to proteins and lipids, the cell supernatant contains extracellular vesicles that can be isolated using an ultracentrifugation protocol, ultrafiltration, or immunoprecipitation technologies with antibody-tagged magnetic beads (Gallina et al. 2015). Extracellular vesicles are classified mainly based on the diameter, molecular weight, and surface markers. Particles with a diameter of 100–1000 nm are called microvesicles (MV) or microparticles, whereas particles with a diameter $<$100 nm are called exosomes (reviewed in Raposo and Stoorvogel 2013). MVs originate from plasma membrane shedding and contain high amounts of phosphatidylserine, selectin, integrin, and CD40. In contrast, exosomes are generated

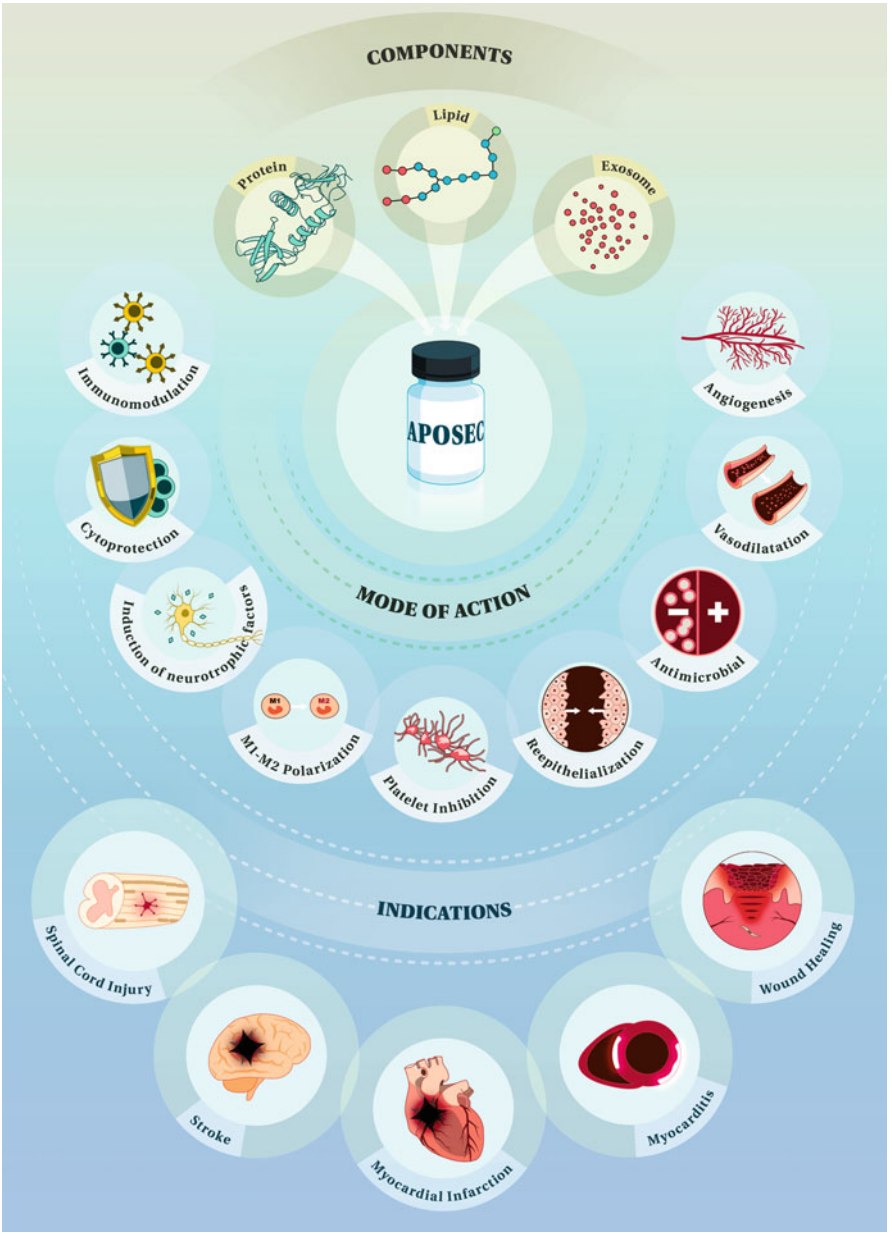


Fig. 2 Components, mode of action, and indication of paracrine factor-based therapies. The cell secretome consists of multiple paracrine factors that can be categorized into different biological classes. The best investigated components are proteins, lipids, and exosomes, which have been shown to exhibit in vitro and in vivo biological activity. Due to the complexity of paracrine factors present in the cell secretome, it is likely that other factors exert biological activity. Paracrine factors derived from apoptotic PBMCs have been shown to induce angiogenesis and vasodilation, exert

intracellularly and stored in the multivesicular bodies until fusion with the plasma membrane and exocytosis of the vesicles. Almost all cell types, including immune cells (Okoye et al. 2014), cancer cells (Costa-Silva et al. 2015), and stem cells (Shabbir et al. 2015), are capable of releasing exosomes. Exosomes contain a set of proteins, lipids, messenger RNA (mRNA), and microRNA (miRNA), all of which contributing to cell-cell signaling (Raposo and Stoorvogel 2013). For a long time, the communication between cells and tissues has been thought to be dependent on the secretion of soluble mediators, including cytokines and chemokines. The discovery of MVs some years ago opened a completely new field of cell-cell communication (Raposo and Stoorvogel 2013). In contrast to cell signaling via single factors, MVs deliver a variety of molecules into other cells, which could strongly influence the cellular function of the recipient cells (Camussi et al. 2010). Importantly, miRNAs that are incorporated into MVs significantly regulate gene expression in the recipient cells (Lee et al. 2012). Recently, the effect of IR on miRNA expression in human PBMCs was reported (Beer et al. 2014). In this study almost 200 miRNAs were found to be significantly regulated by IR at 20 h after irradiation (Beer et al. 2014). Several of these miRNAs are well known to be involved in the regulation of apoptosis and cell proliferation. Ongoing research projects will investigate the contribution of MV-derived miRNAs to tissue regulation. Sophisticated bioinformatics analyses of miRNA-mRNA interactions might help to identify potential therapeutic miRNA. In the same study, Beer et al. characterized EVs released from irradiated and non-irradiated PBMCs. Gamma irradiation induced the release of both exosomes and microparticles and altered the exosomal protein content. Functional analysis revealed that exosomes and proteins are the two major biological components that induce keratinocyte and fibroblast migration and the secretion of pro-angiogenic proteins. The exact contribution of EVs to tissue regeneration, however, remains to be elucidated.

Although the principle components released from apoptotic PBMCs have been characterized, there are questions yet to answer. What is the biologically active component? Which factors interact with each other? Whose separation would abolish the biological activity? Based on the complexity of pathological changes during an ischemic injury, it seems rather plausible to target variable pathological pathways using multifaceted drug compounds, such as apoptotic PBMC-S, instead of focusing on one single aspect. The identification of a specific cause and effect relationship on a cellular level is a matter of systems biology rather than to define a drug compound that is potentially able to treat multiple indications of unmet need.

Fig. 2 (continued) antimicrobial activity, enhance reepithelialization, inhibit platelet coagulation, induce M1-M2 polarization, augment the release of neurotropic factors, exhibit cytoprotective capacities due to the upregulation of anti-apoptotic proteins, and act in an immunomodulatory manner. Based on these biological effects, the PBMC secretome has been successfully tested in animal models to treat acute myocardial infarction, chronic heart failure, myocarditis, skin ulcer, stroke, and spinal cord injury

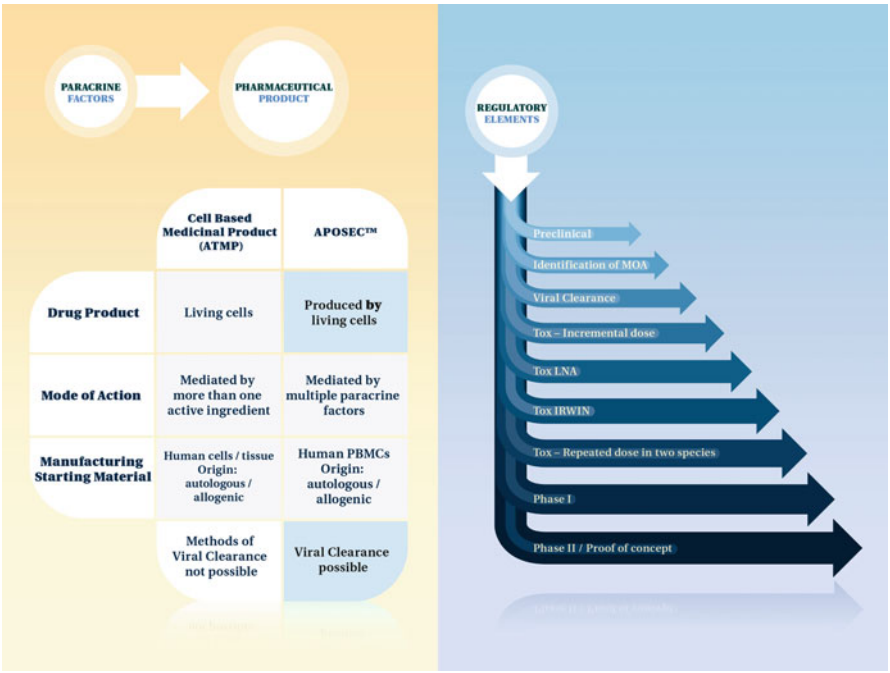


Fig. 3 Regulatory issues for cell-free therapies. To enable regulatory approval for testing paracrine factor-based therapy, several regulatory issues have to be fulfilled. First, cell-free therapies have to be discriminated from cell-based medicinal products (ATMP). ATMPs deal with living cells, whereas paracrine factor-based therapies, such as APOSECTM, contain only factors produced by living cells. In cell therapies, the mode of action is thought to be mediated by the cells or their paracrine factors, whereas in cell-free therapies multiple paracrine factors are thought to exert the biological activity. The starting materials in both therapies are cells of human origin, either autologous or allogeneic. An important difference between these two types of therapies is that viral clearance is not possible in ATMPs, but viral clearance steps can be applied in cell-free therapies such as APOSECTM. In the right side of the figure, the different regulatory steps that have to be performed during the development of a cell-free therapy are depicted, starting from preclinical studies and leading to Phase I/Phase II studies

8 Clinical Utilization of PBMC Paracrine Factors and Regulatory Affairs

When discussing current therapies in regenerative medicine, there are two different types of therapies, consisting of stem cell-based products or recombinant proteins (Fig. 3). The former are so-called advanced therapy medicinal products (ATMPs), products with a substantial manipulation of tissues/cells leading to an alteration of the biological/physiological function (Schneider et al. 2010). Cell production without substantial manipulation is treated differently by the regulatory systems. For example, the in vitro cultivation of cells represents a significant manipulation, whereas harvesting cells for an autologous therapy without cultivation is not a

substantial manipulation. Cell-free recombinant therapies are treated differently by the regulatory offices. In the European Union, the European Medicines Agency (EMA) established a Committee for Advanced Therapies (CAT) for ATMPs, and the Biological Working Party (BWP) is responsible for recombinant protein-based therapies. In the United States, the Food and Drug Administration (FDA) is responsible for the approval of ATMP-based therapies.

Cell-free therapies with multiple paracrine factors produced by living autologous/allogeneic cells fit neither ATMP-based therapy nor recombinant protein therapy. Paracrine factors from apoptotic PBMCs (APOSEC™) are categorized by the authorities as "biologics." The classification as a biologic impacts regulatory requirements. However, the exact management practices during the approval processes for these new classes of therapies remain controversial and are somewhat obscure. Based on our experience, several important issues have to be considered to enable the clinical application of cell-free therapies.

First, it is important to investigate the most likely MOA and mechanism of action. The latter describes the pharmacological effect a substance exerts in the target organism. A drug substance is usually thought to have more or less specific targets, such as receptors or enzymes that are modulated in the presence of the drug (e.g., aspirin, irreversible inhibition of the enzyme cyclooxygenase). In contrast, the MOA characterizes the functional responses at the cellular level that result from a drug therapy. Knowledge of both the mechanism of action and mode of action of a substance helps enhance the achievement of regulatory approval. However, based on our experience in cell-based and paracrine research, a simplification of the monocausal effect of pleiotropic paracrine factors is considered to be little likely the case. We think that cell-free and cell-based therapies have multiple different molecular targets and that only the interactions of these different effects lead to the beneficial clinical impacts observed in multiple preclinical studies.

Second, the final product has to be produced in accordance with good manufacturing practice (GMP) guidelines with validation of all production steps. According to the GMP guidelines, only cell culture media free of indicator dyes (e.g., phenol red) and produced according to the GMP guidelines can be used for the production of the drug substances.

Third, the paracrine factors have to be characterized in terms of appearance, identity, purity, content, biological activity, and stability. In particular, the development of potency assays is mandatory, as they prove the biological activity of each product charge. In addition, the identification of lead product ingredients (e.g., growth factors, cytokines) is recommended to prove batch to batch comparability.

Fourth, stability assays and toxicology studies have to be performed. Toxicology studies include animal studies in two different species with repeated dose testing and the evaluation of any significant immunogenicity of the product.

Finally, and most importantly, the regulatory agencies require implementing two different viral clearance steps before conducting any trials in human beings. However, it is only possible in cell-free products derived from cells. Therapeutic nucleated cells, as defined by ATMP guidelines, are excluded in this mandatory procedure. Possible pathogen-reducing methods range from gamma irradiation and

UV methylene blue inactivation to nanofiltration, pasteurization, precipitation, or vapor heat inactivation (World Health Organization 2004). The method should be selected based on the type of product, the expected pathogen, and the characteristics of the manufacturing process. As shown in Fig. 1, we used UV methylene blue inactivation in combination with gamma irradiation to secure a pathogen-free product in our GMP normalization process.

All of these manufacturing and regulatory steps should be completed successfully before venturing into the clinic. In addition, regulatory approval (e.g., EMA, FDA, AGES), ethics approval, and trial registration have to be obtained.

9 From Bench to Bedside: The Potential Benefit and Safety of Secretome-Based Therapy

Basic science and preclinical research should ultimately aim to transfer knowledge into clinical practice. Though stem cell-based clinical trials at the beginning of the twenty-first century have paved the way for the idea of regenerative medicine, it is now thought that the first clinical trials utilizing PBMC secretome will successfully mediate the human proof of concept in several indications with unmet need. Diabetic foot ulcer, AMI, and spinal cord injury are the first envisioned indications. Ankersmit's group recently finished a Phase I study utilizing GMP PBMC secretome ("Marsyas 1", EudraCT Nr.: 2013-000756-17).

On the way toward clinical application, physicians, non-clinical researchers, and investors have collaborations with reciprocal advantages for each discipline. Currently, clinically oriented researchers and non-clinical scientists are in fast-moving environments that provide great opportunities for both the development and implementation of novel therapeutic modalities (Gabriel 2014). However, closer cooperation between the disciplines is needed to transform basic science into clinical applicability that is in line with GMP guidelines and regulatory constraints. For example, a closer interaction between blood banks with major preclinical driving forces could accelerate scientific progress.

Blood bank and transfusion units should not just follow urgent changes in clinical settings but should be actively incorporated into the working process because they are familiar with GMP policy. They are familiar with handling inspections and are experienced in testing biological samples (Gabriel 2014). Detailed knowledge of the risk involved with transfusion and transplantation, as well as avoiding microbial contamination, is crucial in the setup of cell-free therapies, but these regulations are not well known by medical professionals except for transfusion physicians. Blood banks could establish and run a cell-free therapy unit according to GMPs. Thus, in the future, the cooperation between clinically orientated scientists and blood banks could accelerate the inclusion of investors, as the certification of a validated GMP facility would result in significant (financial) risk reduction. This will eventually introduce a novel chapter in the evolution of regenerative medicine, from cell-based therapies to cell-free therapies, from stem cell therapy to white blood cell therapy, and from limited source therapy (e.g., stem cells) to an easily obtainable cell source (e.g., PBMCs) – a current waste product in transfusion units.

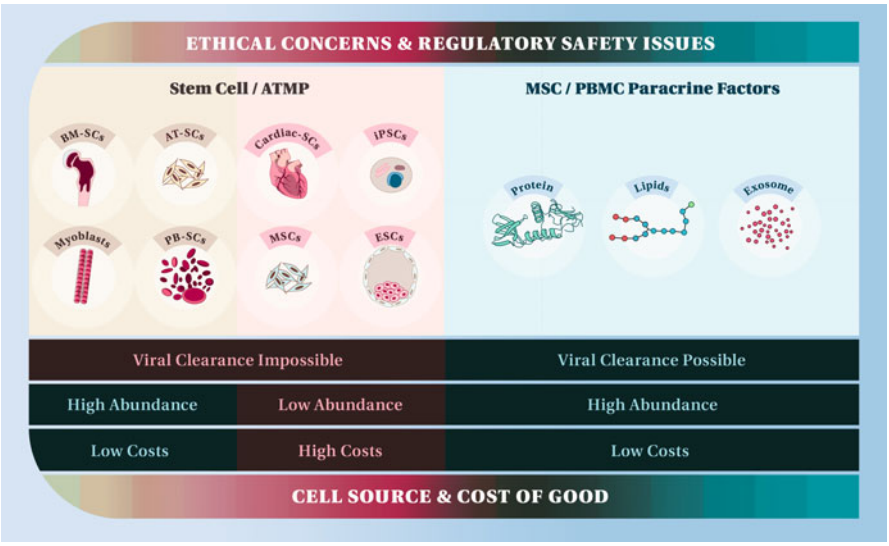


Fig. 4 Ethical, economic, and safety considerations of stem cell-based and paracrine factor-based therapies. In contrast to stem cell-based therapies, paracrine factor-based therapies can be seen as an economic, ethical, and fully acceptable therapeutic strategy lacking significant safety issues and restrictions in production capacity. Viral clearance methods are not possible in stem cell-based therapies containing myoblasts, bone marrow stem cells (BM-SCs), peripheral blood stem cells (PB-SCs), adipose tissue-derived stem cells (AT-SCs), mesenchymal stem cells (MSCs), cardiac stem cells (Cardiac-SCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs). In addition, ESC- and iPSC-based therapies are associated with high costs for cell manufacturing and low cell numbers. Stem cell-based therapies with ESCs, and especially iPSCs, also have ethical and safety concerns because these cells have an embryonic source or bear a risk of malignant transformation. In contrast, paracrine factors derived from PBMCs or MSCs, including exosomes, proteins, and lipids, can be subjected to viral clearance methods, guaranteeing a viral-free medical drug. In addition, paracrine factors, especially from peripheral blood mononuclear cells (PBMCs), can be produced and stored in high amounts with low costs

10 Conclusion

The use of human PBMCs as bioreactors for secretome-based therapies can be seen as an extension of the original concept of stem cell-based therapies to enable clinical applications. A large number of studies of transplanted stem cells have failed to show any significant integration and differentiation of injected cells into mature (cardiac) cells. In addition, there is a considerable loss of injected cells immediately after translation. Using apoptotic PBMC-S instead of stem cells, we could promote tissue repair and regeneration in multiple preclinical indications with the identification of a variety of MOAs. However, as stem cells are rare, their isolation and cultivation in large numbers is neither simple nor practical. Therefore, a more easily obtainable source of cells as the “bioreactor” for the secretome may facilitate the introduction of this new secretome-based medicine.

PBMCs release pleiotropic paracrine factors that augment tissue regeneration, and their usage has the following advantages: I) PBMCs are a current waste product from each red blood donation, so their amount is unrestricted compared to any stem cell population used thus far; II) PBMCs are obtained from mainly healthy persons, avoiding any disease- or age-dependent disadvantage in the PBMC secretome; III) allogeneic samples with pooled PBMC factor from many individuals can be used instead of a strict autologous stem cell therapy approach; and IV) the use of PBMCs does not comprise significant ethical problems, unlike the use of fetal stem cells (Fig. 4). Thus, the PBMC secretome may potentially revolutionize regenerative medicine when appropriate proof of concept studies in humans that meet the requirements of regulating agencies are finished.

Acknowledgments We thank Daniela Leitner for the design of the figures and investors HJA, MP, KF, IP, and HPH for their financial support. Research on the PBMC secretome was financed by the Christian Doppler Laboratory for Diagnosis & Regeneration in Thoracic Diseases (2009–2015) under group leader HJA and FFG-Grant "APOSEC" (#852748; 2015–2018). We also are indebted to Prof. Walter Klepetko and Prof. Michael Gnant, as well as all of the diploma/PhD students and scientific colleagues involved in the studies. Special thanks to Prof. Meinrad Peterlik, former head of the Department of Pathophysiology at the medical faculty of the University of Vienna (1984–2006).

Disclosure Statement The Medical University of Vienna has claimed financial interest. Aposcience AG holds patents related to this work (EP20080450198 and EP20080450199). HJA is a shareholder of Aposcience AG. All other authors declare no potential conflicts of interest.

References

Altmann P, Mildner M, Haider T et al (2014) Secretomes of apoptotic mononuclear cells ameliorate neurological damage in rats with focal ischemia. F1000Res 3:131

Ankersmit H, Moser B, Zuckermann A et al (2002) Activation-induced T cell death, and aberrant T cell activation via TNFR1 and CD95-CD95 ligand pathway in stable cardiac transplant recipients. Clin Exp Immunol 128:175–180

Ankersmit HJ, Hoetzenecker K, Dietl W et al (2009) Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium. Eur J Clin Investig 39:445–456

Assmus B, Schachinger V, Teupe C et al (2002) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 106:3009–3017

Balsam LB, Wagers AJ, Christensen JL et al (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428:668–673

Beer L, Seemann R, Ristl R et al (2014) High dose ionizing radiation regulates micro RNA and gene expression changes in human peripheral blood mononuclear cells. BMC Genomics 15:814

Beer L, Zimmermann M, Mitterbauer A et al (2015) Analysis of the secretome of apoptotic peripheral blood mononuclear cells: impact of released proteins and exosomes for tissue regeneration. Sci Rep 5:16662

Behfar A, Crespo-Diaz R, Terzic A et al (2014) Cell therapy for cardiac repair – lessons from clinical trials. Nat Rev Cardiol 11:232–246

Beltrami AP, Urbanek K, Kajstura J et al (2001) Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 344:1750–1757

Bergmann O, Bhardwaj RD, Bernard S et al (2009) Evidence for cardiomyocyte renewal in humans. Science (New York, NY) 324:98–102
Bergmann O, Zdunek S, Felker A et al (2015) Dynamics of cell generation and turnover in the human heart. Cell 161:1566–1575
Boland K, Flanagan L, Prehn JH (2013) Paracrine control of tissue regeneration and cell proliferation by Caspase-3. Cell Death Dis 4:e725
Camussi G, Deregibus MC, Bruno S et al (2010) Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int 78:838–848
Chen SL, Fang WW, Ye F et al (2004) Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 94:92–95
Costa-Silva B, Aiello NM, Ocean AJ et al (2015) Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol 17:816–826
David S, Zarruk JG, Ghasemlou N (2012) Inflammatory pathways in spinal cord injury. Int Rev Neurobiol 106:127–152
Fadok VA, Bratton DL, Konowal A et al (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 101:890–898
Fisher SA, Doree C, Mathur A et al (2015) Meta-analysis of cell therapy trials for patients with heart failure. Circ Res 116:1361–1377
Fisher SA, Doree C, Taggart DP et al (2016) Cell therapy for heart disease: trial sequential analyses of two Cochrane reviews. Clin Pharmacol Ther 100:88–101
Földes I, Varkonyi S, Solti F et al (1970) Effect of antilymphocyte serum on experimental myocardial infarction. Experientia 26:741–743
Gabriel C (2014) How to establish and run a cell therapy unit in a blood bank. ISBT Sci Ser 9:155–159
Gallina C, Turinetto V, Giachino C (2015) A new paradigm in cardiac regeneration: the mesenchymal stem cell secretome. Stem Cells Int 2015:765846
Gnecchi M, Melo LG (2009) Bone marrow-derived mesenchymal stem cells: isolation, expansion, characterization, viral transduction, and production of conditioned medium. Stem Cells Regen Med 482:281–294
Gnecchi M, Zhang Z, Ni A et al (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103:1204–1219
Gnecchi M, Danieli P, Malpasso G et al (2016) Paracrine mechanisms of mesenchymal stem cells in tissue repair. Mesenchymal Stem Cells Methods Protocol 1416:123–146
Grau A, Tabib A, Grau I et al (2015) Apoptotic cells induce NF-kappaB and inflammasome negative signaling. PLoS One 10:e0122440
Gray M, Miles K, Salter D et al (2007) Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. Proc Natl Acad Sci U S A 104:14080–14085
Gyöngyösi M, Wojakowski W, Lemarchand P et al (2015) Meta-analysis of cell-based CaRdiac stUdiEs (ACCRUE) in patients with acute myocardial infarction based on individual patient data. Circ Res 116:1346–1360
Hacker S, Mittermayr R, Nickl S et al (2016) Paracrine factors from irradiated peripheral blood mononuclear cells improve skin regeneration and angiogenesis in a porcine burn model. Sci Rep 6:25168
Haider T, Hoftberger R, Ruger B et al (2015) The secretome of apoptotic human peripheral blood mononuclear cells attenuates secondary damage following spinal cord injury in rats. Exp Neurol 267:230–242
Hatzistergos KE, Quevedo H, Oskouei BN et al (2010) Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 107:913–922
Hodgkinson CP, Bareja A, Gomez JA et al (2016) Emerging concepts in paracrine mechanisms in regenerative cardiovascular medicine and biology. Circ Res 118:95–107
Hoetzenecker K, Assinger A, Lichtenauer M et al (2012) Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute

myocardial infarction model: role of platelet aggregation and vasodilation. Basic Res Cardiol 107:292
Hoetzenecker K, Zimmermann M, Hoetzenecker W et al (2015) Mononuclear cell secretome protects from experimental autoimmune myocarditis. Eur Heart J 36:676–685
Holzinger C, Zuckermann A, Kopp C et al (1994) Treatment of non-healing skin ulcers with autologous activated mononuclear cells. Eur J Vasc Surg 8:351–356
Kasiri MM, Beer L, Nemec L et al (2016) Dying blood mononuclear cell secretome exerts antimicrobial activity. Eur J Clin Investig 46:853–863
Kocher AA, Schuster MD, Szabolcs MJ et al (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
Korf-Klingebiel M, Kempf T, Sauer T et al (2008) Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. Eur Heart J 29:2851–2858
Kwak BR, Mach F (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11:367
Laflamme MA, Myerson D, Saffitz JE et al (2002) Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res 90:634–640
Lauber K, Bohn E, Krober SM et al (2003) Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell 113:717–730
Lee Y, El Andaloussi S, Wood MJ (2012) Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Hum Mol Genet 21:R125–RR34
Lichtenauer M, Mildner M, Baumgartner A et al (2011a) Intravenous and intramyocardial injection of apoptotic white blood cell suspensions prevents ventricular remodelling by increasing elastin expression in cardiac scar tissue after myocardial infarction. Basic Res Cardiol 106:645–655
Lichtenauer M, Mildner M, Hoetzenecker K et al (2011b) Secretome of apoptotic peripheral blood cells (APOSEC) confers cytoprotection to cardiomyocytes and inhibits tissue remodelling after acute myocardial infarction: a preclinical study. Basic Res Cardiol 106:1283–1297
Lichtenauer M, Mildner M, Werba G et al (2012) Anti-thymocyte globulin induces neoangiogenesis and preserves cardiac function after experimental myocardial infarction. PLoS One 7:e52101
Losordo DW, Schatz RA, White CJ et al (2007) Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. Circulation 115:3165–3172
Mansour S, Roy DC, Bouchard V et al (2010) COMPARE-AMI trial: comparison of intracoronary injection of CD133+ bone marrow stem cells to placebo in patients after acute myocardial infarction and left ventricular dysfunction: study rationale and design. J Cardiovasc Transl Res 3:153–159
Maximow AA (1909) Der lymphozyt als gemeinsame stammzelle der verschiedenen blutelement in der embryonalen entwicklung und im postfetalen leben der säugetiere. Folia Haematol 8:125–134
Metchnikoff II (2004) The prolongation of life: optimistic studies. Springer Publishing Company, New York
Mildner M, Hacker S, Haider T et al (2013) Secretome of peripheral blood mononuclear cells enhances wound healing. PLoS One 8:e60103
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 104:1643–1648
Murry CE, Soonpaa MH, Reinecke H et al (2004) Haematopoietic stem cells do not trans-differentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668
Nguyen PK, Lan F, Wang Y et al (2011) Imaging: guiding the clinical translation of cardiac stem cell therapy. Circ Res 109:962–979
Niehans P (1952) 20 Jahre Zellular-Therapie. Urban & Schwarzenberg, Berlin

Notley CA, Brown MA, McGovern JL et al (2015) Engulfment of activated apoptotic cells abolishes TGF-beta-mediated immunoregulation via the induction of IL-6. J Immunol 194:1621–1627
Okoye IS, Coomes SM, Pelly VS et al (2014) MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. Immunity 41:89–103
Orlic D, Kajstura J, Chimenti S et al (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705
Pavo N, Zimmermann M, Pils D et al (2014) Long-acting beneficial effect of percutaneously intramyocardially delivered secretome of apoptotic peripheral blood cells on porcine chronic ischemic left ventricular dysfunction. Biomaterials 35:3541–3550
Perruche S, Saas P, Chen W (2009) Apoptotic cell-mediated suppression of streptococcal cell wall-induced arthritis is associated with alteration of macrophage function and local regulatory T-cell increase: a potential cell-based therapy? Arthritis Res Ther 11:R104
Premer C, Blum A, Bellio MA et al (2015) Allogeneic mesenchymal stem cells restore endothelial function in heart failure by stimulating endothelial progenitor cells. EBio Med 2:467–475
Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200:373–383
Rietschel H (1954) Gefahren der Frischzellentherapie. DMW Deut Med Wochenschr 79:1671–1673
Saas P, Daguindau E, Perruche S (2016) Concise review: apoptotic cell-based therapies -rationale, preclinical results and future clinical developments. Stem Cells (Dayton, Ohio) 34:809–819
Schneider CK, Salmikangas P, Jilma B et al (2010) Challenges with advanced therapy medicinal products and how to meet them. Nat Rev Drug Discov 9:195–201
Shabbir A, Cox A, Rodriguez-Menocal L et al (2015) Mesenchymal stem cell exosomes induce proliferation and migration of normal and chronic wound fibroblasts, and enhance angiogenesis in vitro. Stem Cells Dev 24:1635–1647
Thum T, Bauersachs J, Poole-Wilson PA et al (2005) The dying stem cell hypothesis: immune modulation as a novel mechanism for progenitor cell therapy in cardiac muscle. J Am Coll Cardiol 46:1799–1802
Wollert KC, Drexler H (2005) Mesenchymal stem cells for myocardial infarction promises and pitfalls. Circulation 112:151–153
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 364:141–148
World Health Organization (2004) Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products. WHO Tech Rep Ser 924:150–224

Part V

Learning from Embryogenesis and Evolution

Basics of Self-Regeneration

Rita Aires, Sean D. Keeley, and Tatiana Sandoval-Guzmán

Contents

R. Aires · S. D. Keeley
CRTD Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany
e-mail: rita.aires@tu-dresden.de; sean.keeley@mailbox.tu-dresden.de

T. Sandoval-Guzmán (✉)
CRTD Center for Regenerative Therapies, Technische Universität Dresden, Dresden, Germany

Center for Healthy Aging, Medical Faculty Carl Gustav Carus Technische Universität Dresden, Dresden, Germany
e-mail: tatiana.sandoval_guzman@tu-dresden.de

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_66

Abstract

Regeneration is a fascinating phenomenon spanning several levels of complexity, allowing organisms to repair, renew, and reconstitute missing or damaged parts. Despite significant efforts to understand regeneration, the lack of adequate technology has slowed advances in the field until very recently. Since the beginning of the eighteenth century, scientists have looked toward animal models while striving to dissect the cellular and molecular pathways that drive regeneration. Although we are still far from the day that humans could regenerate an appendage, several mechanisms used by other animal species have been uncovered. This knowledge brings us closer to their potential transfer to humans, enabling the repair of specific tissues.

Even though regeneration occurs in many organisms, the extent of this trait varies enormously: planarian worms can regenerate their whole body and are even able to create two new worms from a bisected individual, whereas mammals can only regenerate specific parts of an organ or tissue, such as the very distal tip of a digit. Among vertebrates, however, salamanders are able to regenerate complex structures such limbs or tails, making them the object of intense scientific research. This chapter addresses limb regeneration using one specific species of salamander – the axolotl – as an example of a complex structure that is re-formed from cells of different embryonic origins. It also discusses the advances in technology that allow the pursuit and answering of important questions within this topic, and includes a brief look into the future and what remains to be explored in the field.

1 Introduction

Regeneration is defined as replacing a lost part with a similar and functional copy. However, this definition does not fully encompass the broad range of biological responses to injury or continuous tissue wear that exists within the animal kingdom. All major phylogenetic groups contain species capable of regeneration, but only some animals possess the remarkable ability to replace a considerable portion of their body or organs. In vertebrates for example, this ability varies greatly, ranging from simple tissue repair, to organ regeneration, and, finally, to the flawless substitution of entire complex body structures such as a limb or tail. More impressive mechanisms of regeneration are exhibited by freshwater cnidarians and flatworms, such as planaria, that can regenerate their whole-body plan from a small tissue fragment. The study of innate regenerative mechanisms in these animal species, and particularly their contrast to mammalian regeneration, could provide the basis to further control regenerative processes.

Due to the varying levels of regenerative potential across species, the ability to regenerate has often been subdivided into distinct classes. For example, the continuous replacement of worn out tissue is classified as physiological regeneration, in which cell turnover maintains tissue homeostasis in the body. Two well-studied examples of physiological regeneration are the epidermis and the epithelial cells in the gut (Wells and Watt 2018). Alternatively, there is also restorative regeneration triggered by trauma, which will be the focus of this chapter. Restorative regeneration was once defined by Thomas Hunt Morgan as consisting of epimorphic regeneration – the creation of new material by proliferation – and its counterpart, morphallaxis – the remodeling of existing tissue to substitute missing parts in the absence of proliferation (Morgan 1901). However, this definition has continued to evolve. With the advancement of techniques and a growing mass of literature, it is becoming clear that some organisms combine aspects of both mechanisms and that this historical distinction does not define the strategy employed by one species, or even an organ.

The modern concept of regeneration now integrates not only the sequential steps that direct cells to form tissues but also the communication that allows these cells to coordinate their behavior (Sandoval-Guzmán and Currie 2018). Furthermore, it incorporates the importance of the wound epithelium and immune system as necessary building blocks in the creation of new tissue. The challenge is to integrate the chronological and topological coordination of chemical and physical cues that drive remodeling of preexisting tissue, the creation of new material, and the integration of both to functionally reconstitute the missing parts.

A large scientific effort has been concentrated on studying the process of recruitment of regeneration-competent cells arising from the tissues at the injury site, which in some cases lead to the formation of a transient structure called the blastema. The blastema contains the progenitor cells that produce, both in numbers and type, the necessary cells that will respond to chemical, bioelectrical, and physical cues to pattern the lost tissue or organ. In vertebrates, the origin of these cells and their lineage restriction is often conserved, but there are important differences in how each lineage will contribute to the regenerate (Kragl et al. 2009; Lehoczky et al. 2011; Rinkevich et al. 2012). Among vertebrates, the teleost fish and amphibians are the best examples of animals with high regenerative potential. Zebrafish can regenerate the spinal cord, fins, and even part of the heart and brain. Urodele and anuran amphibians are the preferred choice of tetrapods to study appendage and jaw regeneration. These animal models are easy to breed and maintain in laboratory, and have been the basis of a plethora of studies that increased our understanding of self-regeneration. Despite the high gene homology between these regenerative species and mammals, the difference in regenerative potential is outstanding. However, although mammalian regenerative capacity is comparatively poorer, it has been extensively studied in murine models. Adult mice can regenerate the distal part of the third phalanx (P3), and, therefore, the digit tip has become a standard assay for appendage regeneration. A more recent mammalian model is the African spiny mouse, a curious rodent that is capable of healing its skin without forming a scar and can regenerate tears and holes in its ear (Seifert et al. 2012).

Human regeneration, on the other hand, has been harder to define than amphibian regeneration, probably due to the lack of consistent evidence and the reticence to acknowledge that humans, albeit limited, do have regenerative capacity. Similar to mice, humans can regenerate the tips of the digits, provided enough of a nail piece remains. Additionally, liver regeneration after surgical or chemical injury is a striking example of regenerative potential, where only a quarter of the organ is enough to fully restore the missing part by activating the proliferation of hepatocytes.

One relevant aspect of the study of regeneration is the influence of aging on the regenerative abilities of some species. Older zebrafish regenerate their fins poorly when compared to younger specimens (Anchelin et al. 2011), and mammals show substantial decreases in both physiological and restorative regeneration over time (Brunauer and Muneoka 2018; Yun 2015). Specific examples of age-limited mammalian regeneration have been documented in mice. The heart, for example, can regenerate a partial resection within the initial days after birth, but this ability is lost after 1 week (Porrello et al. 2011). This shows that mammals have an intrinsic potential to regenerate, which is lost or inhibited with time. The aim of regenerative medicine therefore is to rescue the progression of healing to a regenerative outcome. Although many animal and plant species show various degrees and mechanisms of regeneration, this chapter will only briefly describe a few of them, focusing instead on salamander appendage regeneration and how we can combine or translate our studies toward the development of human regenerative medicine.

2 Brief History of Research in Regeneration

Ever since ancient times there has been a natural curiosity and a need to understand how our body works and how diseases can be cured. Even though no precise record exists on when the first healing traditions started, it seems that the study of the body has concerned humans even in prehistorical societies. Upper Paleolithic cave paintings – which probably constitute the first depictions of human amputations – show hands with missing fingers recently suggested as purposely removed with religious connotations, emphasizing the value of losing parts of the body (McCauley et al. 2018). Likewise, Greek mythology is full of heroes and creatures with enormous regenerative traits. As a damaged organ or the loss of a body part significantly compromises well-being and the ability to function in daily life, it is no surprise that the drive to find answers and regenerate what is missing is as appealing now as it was back then. As a result, modern medicine devotes an increasing amount of economic and human resources to heal diseases and cure illness, as well as to find new approaches to ease pain and offer a good quality of life to those suffering from disruptions of bodily functions.

Regeneration transitioned from being a curiosity of nature to becoming a part of rigorous scientific study at the beginning of the eighteenth century. In 1712, René-Antoine Ferchault de Réaumur (1683–1757) presented the first systematic scientific description of animal regeneration to the French Academy by describing limb

regeneration of the crayfish. To the Academy, limb regeneration was introduced as a specific ability of crayfish, and believed to be rather unusual in other species. In the context of the then philosophical debate, his description was coined as "reproduction of legs and shells of crayfish" (Nachtomy and Smith 2014). During that time preformationist views dominated the scientific debate, and Réaumur hypothesized that the regenerating limbs arose from the expansion of tiny preformed limbs that resided within the mature structure. A series of seminal scientific works then followed his detailed study, which described the phenomenon of regeneration across many animal phyla.

In 1744, Abraham Trembley, while tutoring the sons of his benefactor in Hague, became curious of an organism that he could not define as either a plant or an animal. By cutting what is now known as a Hydra in half, he assumed that the organism would be a plant if it regenerated and, conversely, an animal if it did not. Although both parts regenerated into two similar hydras and, thus, the conclusion should have been clear, the complex behavior displayed by the organism made Trembley reevaluate his assumptions and consider the Hydra to be an animal – a conclusion similarly drawn by Réaumur after Trembley described to him his findings over correspondence. And so, from the ponds of the Hague, Abraham Trembley founded the modern study of regeneration (Dinsmore 2007).

In 1745, Charles Bonnet, inspired by reading the findings of Trembley and Réaumur, added the segmented worms (annelids) to the list of animals that re-form a missing part. His findings elevated regeneration from being an isolated and unique phenomenon of selected animals, to a widened concept that pressured natural researchers to reconcile existing ideas of life precepts. In an attempt to reconcile philosophical concepts on the formation of life, it was assumed that regeneration was a trait pertaining to animals that are equipped with germs purposed for regeneration. In 1766, the work of Peter Simon Pallas describing flatworm regeneration added to the momentum and turned the attention of others to the systematic study of regeneration in planarians.

Finally, to Lazzaro Spallanzani (1729–1799) we owe the beginning of the field of amphibian limb and tail regeneration. His studies on different species published in 1768 – *Prodromo di un opera da imprimersi sopra la riproduzioni animali* – aimed to describe the animals' reproduction but, in addition, it provided an outline for regeneration in vertebrates. Spallanzani's observations on the complexity of limb regeneration made him conclude that regeneration could not be mere elongation of existing tissues, thus rejecting the prevalent preformationist view. As salamanders are the only tetrapods that can fully regenerate limbs and the tail as adults, they have since become popular model organisms for the study of regeneration.

From early development, organisms embark on a life-long balance of repairing tissue by constant renewing and replenishing of cells. Regeneration is used for many different purposes, such as a form of reproduction, like in planarians, or as a means to escape predators, like in lizards and spiny mice. Yet others, like salamanders, choose to spend quite a significant proportion of their biological resources to re-form large portions of complex structures without an obvious purpose. Ultimately, the study of several animal species for more than 300 years has brought us closer to

understanding the cellular origin of the regenerated tissue and many of the signals orchestrating the regenerative process.

3 Animals Models of Regeneration

The term "physiological regeneration" has been applied to a wide variety of processes that do not even share a common mechanism. The natural replacement of blood cells and the constant turnover of epidermal cells, for example, are certainly not mechanistically comparable to repairing a damaged bone, as the activated mechanisms and cellular behaviors vary broadly. Similarly, the cellular source to replace a head or tail in a planarian differs greatly from the ones needed to reconstitute a missing tail in a salamander. However, important factors and signaling pathways are shared, as well as the integration of environmental cues into the regenerative process.

Common genetic networks in regenerative organisms or organs, as well as their differences from regeneration-incompetent tissues, can be established through extensive comparative analyses. Only then can modern medicine move on to answer the most pressing questions in regenerative medicine: How can we instruct our own cells to heal and reconstitute a damaged part? How can we instruct our own cells to accept xeno-transplantations or biomaterials?

Although there are remarkable examples of animal regeneration, and equally remarkable work from researchers around the world, this chapter will only briefly describe four species: two invertebrates – the hydra and planarian – that can regenerate fully from very small pieces, and two vertebrates – the zebrafish and the salamander (Fig. 1). The chapter will end with a word on mammalian regeneration and the challenges in regenerative medicine.

3.1 Hydra

The freshwater cnidarian polyp – the Hydra – is one of the most extraordinary examples of regenerative abilities in the entire animal kingdom. If a Hydra is cut in half, the foot end will regenerate a head and the head will regenerate a foot, resulting in two smaller Hydras. Furthermore, if a Hydra is fragmented into several pieces, the pieces in the center will produce both a head and foot. Even aggregates of cells that have been previously dissociated can regenerate a whole animal within a couple of days (Gierer et al. 1972).

The Hydra is a predatory animal with a tubular body no bigger than 10 mm, displaying a basal structure, or foot, in one of its extremities, and a mouth opening surrounded by tentacles on the opposite side. While lacking an apparent brain, hydras are equipped with a nervous system that controls their behavior and predatory movements. Typically, these organisms reproduce by budding off, although some may reproduce sexually.

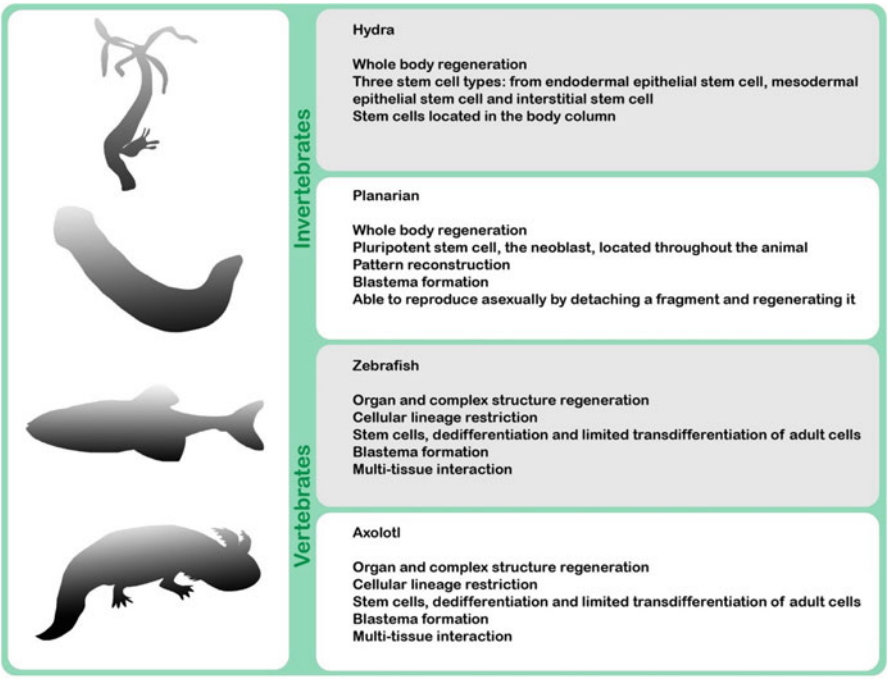


Fig. 1 Nonmammalian models of regeneration: hydra, planarian, zebrafish, and axolotl. A brief comparison of their regenerative abilities and cellular sources

The Hydra has a diploblastic developmental origin, which means that its body wall is composed of two epithelial cell layers, designated as the ectodermal layer and the endodermal layer. The regenerative abilities in hydras are mediated by three types of stem cells that maintain tissue homeostasis: the ectodermal epithelial stem cell, the endodermal epithelial stem cell, and the interstitial stem cell. These three types of stem cells are mitotically active and distributed along the body column, and all three types can differentiate into one or more cell types during regeneration, although each stem cell line cannot replace either of the other two (Galliot and Ghila 2010).

Stemming from Morgan's classical definition of morphallactic regeneration in Hydra, a more plastic definition of regeneration has been proposed. While cell division is not essential – indeed, even when mitosis is inhibited a hydra still regenerates (Park et al. 1970) – it has been shown that adult stem cells paused in G2 will begin readily dividing upon injury. This has lead researchers to propose the mid-gastric region as a pro-blastema area with a sustained proliferative rate (Buzgariu et al. 2018). In fact, this dual ability to reaccommodate cells without proliferation or create new cells to regenerate makes the Hydra an ideal model to study morphogenesis (Braun and Keren 2018).

Later stages of regeneration in this organism are known to reactivate important developmental programs, such as the Wnt pathway, a common trait among

regenerative organisms. The relative simplicity of the Hydra and the plasticity of its cells make it a powerful system to study self-organization in a living organism (Fig. 1). In 2010, the genome of *Hydra magnipapillata* was published (Chapman et al. 2010), adding to the genetic toolkit that makes Hydra a tractable system for regulation of stemness.

3.2 Planarian

A small platyhelminth (flatworm), the planarian has an impressive regenerative capability. In fact, a whole individual can be regenerated from just a tiny piece of the worm; and when planarians are cut in half, regeneration occurs from both wound sites to reconstitute their respective missing body sections. Out of all the planarians, *Schmidtea mediterranea* and *Dugesia japonica* are the most studied species, since both show robust regeneration capabilities (Ivankovic et al. 2019; Newmark and Sánchez Alvarado 2002). However, other species of planarians have more restricted abilities, or even none at all. This diversity provides unparalleled opportunities for comparative studies among species and an accurate description of roadblocks that inhibit successful regeneration in high- and low-regenerating organisms of the same taxa.

Planarians are bilaterally symmetric and develop from all three germ layers (endoderm, ectoderm, and mesoderm). They develop a brain and eye cups, and feed through a ventral mouth opening that continues through a pharynx and into an intestinal system.

The source of regenerative cells in planarians is a population of stem cells called neoblasts. This type of adult somatic stem cell is found throughout the mesenchyme of its body (Baguñà 2012) and is the only mitotic cell type in planarians. They can be distinguished from somatic cells by their morphology and their ability to form any tissue type of the worm – including the heart, brain, and digestive system (Rink 2013; Sanchez 2006; Reddien et al. 2005).

The pluripotency of neoblasts was elegantly confirmed by transplantation of one single neoblast into an irradiated animal. The transplanted neoblast could successfully regenerate the original organism and become an individual carrying the donor genotype; thus, these cells have been defined as clonogenic neoblasts (Wagner et al. 2011). Their pluripotency and high mitotic rate set them apart from other stem cells in the animal kingdom, where lineage restriction defines the cellular fate. However, the molecular factors responsible for the neoblasts' pluripotency, as well as what signals attract neoblasts into the wound, remain unknown.

Some evidence is now pointing to the existence of a subset of specialized neoblasts that are defined by their position and environmental niche (Reddien 2013). The importance of positional information is extensively studied in planarians, where molecular gradients provide the spatial context to pattern missing tissue. A posterior morphogenetic gradient driven by a Wnt gradient (Stückemann et al. 2017) antagonizes an anterior gradient, shaping the antero-posterior axis (AP) (Iglesias et al. 2008). Additionally, perturbations of BMP signaling have been known to

ventralize the animal, which indicates that the dorso-ventral (DV) axis is defined by a BMP signaling gradient (Gaviño and Reddien 2011). Further molecular fine-tuning of the body plan is achieved by the regionalized expression of multiple ligands and receptors belonging to the main signaling pathways (Reddien 2018). Upon injury, the progeny of mitotic neoblasts will form a blastema, which consists of a mass of differentiating cells that reform the tissue (Newmark and Sánchez Alvarado 2000). Ultimately, the signaling gradients that pattern and maintain the anatomy of the planarian will play a key role in regeneration, as they will eventually be reused when regenerating missing tissue pieces.

As recently reviewed, polarity cues, in addition to tissue-resident positional information, may generate a unique signaling milieu at the wound site that will influence cell fate specification of both arriving neoblasts and their progeny (Ivankovic et al. 2019; Reddien 2018).

3.3 Zebrafish

Unlike planarians and hydra, vertebrates do not regenerate the whole body, but rather individual organs and other body structures. However, vertebrate models have provided invaluable knowledge on the heterogeneity of behavior and plasticity of cells when exposed to injury signals. Teleost fish, like the goldfish, trout, tilapia, and zebrafish, make up the vast majority of all fish species. Among these, the zebrafish (*Danio rerio*) has become a genetic model predominately used for regeneration and developmental studies. The advantages of this species include the availability of genetic and genomic tools, easy maintenance in the laboratory, accessibility for high throughput screening, and fast external development (Gemberling et al. 2013; Marques et al. 2019; Sehring et al. 2016). Numerous studies have described regeneration of the central nervous system (Becker and Becker 2008; Kroehne et al. 2011), retina, heart (Poss et al. 2002), fins, and internal organs.

One key difference between invertebrates and vertebrates is the lineage restriction of the cells that contribute to regeneration. Contrary to planarians, which rely on pluripotent cells, the major mechanisms of cellular contribution to regeneration in vertebrates are the proliferation of existing cell types, dedifferentiation, transdifferentiation, and the differentiation of tissue specific stem cells.

The caudal fin of zebrafish is regenerated through an organized blastema, the cellular components of which are restricted by the potential of the mature cells contributing to it – including epidermal tissue, osteoblasts, and fibroblasts (Stewart and Stankunas 2012). The bone, for example, is replaced through dedifferentiation of osteoblasts to a progenitor-like state, downregulation of the expression of *Osteocalcin*, a late differentiating marker of osteoblasts, and upregulation of the expression of pre-osteoblast marker *Runx2* (Knopf et al. 2011). Osteoblast dedifferentiation is similarly responsible for skull regeneration (Geurtzen et al. 2014). Although osteoblast dedifferentiation seems to be a conserved mechanism used to replace bone in zebrafish, ablating them before regeneration triggers the de novo formation of osteoblasts from other sources (Singh et al. 2012).

The heart is one of the most studied injuries in zebrafish. Preexisting cardiomyocytes dedifferentiate and then further proliferate to regenerate the damaged area (Jopling et al. 2010; Kikuchi et al. 2010). Within 2 months, 20% of the heart can be regenerated without a visible scar (Poss et al. 2002).

Finally, regeneration driven by tissue-specific stem cells occurs in the nervous system from radial glial cells (Kroehne et al. 2011), and in skeletal muscle by satellite cells (Berberoglu et al. 2017). The diversity of cellular types in vertebrates has added layers of complexity to the study of the cellular response to regeneration. However, with recently developed technology, individual cells can be dissected out by their transcriptome, morphology, and physical properties. Clonal analysis of cells within the fin blastema has emphasized the heterogeneity of individual cells in regards to their proliferative capacity (Tornini et al. 2016). Research on vertebrates such as zebrafish highlights the multiple mechanisms utilized in just one organism (Fig. 1), the adaptability to use multiple cell sources for regeneration, and the redundancy of mechanisms that ultimately ensure a functional repair.

3.4 Salamanders

Among vertebrates, Urodele amphibians – or salamanders – have no rival concerning regenerative abilities. The myths surrounding these creatures are varied and extensive, existing in cultures all over the world. Within Europe, folklore described how salamanders were beings born from fire, able to survive within flames and extinguish them, while woodblock paintings from Japan depict the creatures growing to stupendous sizes (Satoh et al. 2015). Similarly, the peculiarities of the Mexican salamander, the axolotl (*Ambystoma mexicanum*), also inspired incredible tales in the societies that lived around it. Originally from the lake Xochimilco in central Mexico, the axolotl was remarkable due to it remaining aquatic and neotenic, that is, keeping their juvenile features yet achieving sexual maturity. These extraordinary features inspired legends, such as it being one of the disguises taken by an ancient Aztec god while hiding on earth (Bartra and Viñas 2011), and promises of healing properties by consuming parts of its body (Rabiela 1998). However, nowadays the axolotl is believed to be nearly extinct in its original habitat, and the once "Royalty's delicacy" is mainly preserved in captivity.

The first news outside of the Aztec empire concerning the axolotl came from the Spanish conquerors. Doctors and priests alike were fascinated by it, its neoteny, and the legends preceding it. It comes as no surprise, thus, that this fascination among people with the axolotl continued following its dissemination out of the Americas, particularly after the first documented report regarding its regenerative capabilities which described that, in attempts to induce metamorphosis, Auguste Duméril (1812–1870) dissected the gills but they kept growing back (Bartra and Viñas 2011). It was later found that the axolotl and other salamanders were able to regenerate very complex structures, such as the tail, limbs, and the brain. This spectacular regenerative ability has continued to be a focus of worldwide research to this day. Although regeneration has been found to occur in very different organisms – each one using

dramatically different mechanisms that proceed at significantly different rates – due to its history and structural complexity, the salamander has always been at the forefront of regeneration research. In the end, different strategies are used to achieve successful regeneration, either at the tissue level or for complex structures. Together, all animal models currently used in regeneration research – including emerging model organisms arising from serendipitous discovery, tool availability, or just mere curiosity – provide a plethora of data that will most likely uncover fundamental insights into the general and specific mechanisms of regeneration used throughout the animal kingdom. Translation to human therapies of regeneration can, thus, rely on such diverse mechanisms in order to find the most adaptable and successful ones.

4 Salamander Evolution

Salamanders belong to the order Urodela, which together with frogs (Anura) and caecilians (Gymnophiona) constitute the extant orders of modern amphibians. However, while all three orders share a phylogenetic relationship separating them from amniotes, the exact way in which these taxa diverged from each other is shrouded in mystery and is the subject of much debate (Carroll 2007). It is generally thought that the lineage leading to Gymnophiona was the first to split off (Fig. 2), but whether these three groups derived from a single Paleozoic amphibian ancestor or from multiple distinct ones is currently unknown (Carroll 2007; Shen et al. 2013). This is partly due to the timescale involved, with fossils of species belonging to the Urodela suborder Cryptobranchoidea – which contains many of the modern giant salamanders – being dated to the Middle Jurassic (165 MYA), indicating that the salamander lineage had already become distinct from other amphibians by this time (Gao and Shubin 2003). This difficulty is further compounded by the current lack of fossils predating these throughout the Triassic period, leaving a large gap in the fossil record between the Jurassic and Permian periods (Brockes 2015).

Within the Permian period, however, the fossil record is resumed. Various fossils from the Paleozoic clade of amphibians Temnospondyls already display characteristics associated with modern salamanders, such as preaxial dominant limb development (Fröbisch and Shubin 2011) and limb regeneration (Fröbisch et al. 2014). These findings helped to identify the traits that may be ancestral to all modern salamanders, which nowadays consists of ten families (Fig. 2). Of these, the ones most commonly used today in the study of regeneration are Salamandridae, which contains all newt species, and Ambystomatidae, containing the axolotl along with other species of mole salamanders.

4.1 Axolotl Evolution

The difficulties concerning the establishment of proper phylogenetic relationships within salamander species are not limited to the extinct ones, and the axolotl is a clear example of this. The axolotl is a member of a species complex within

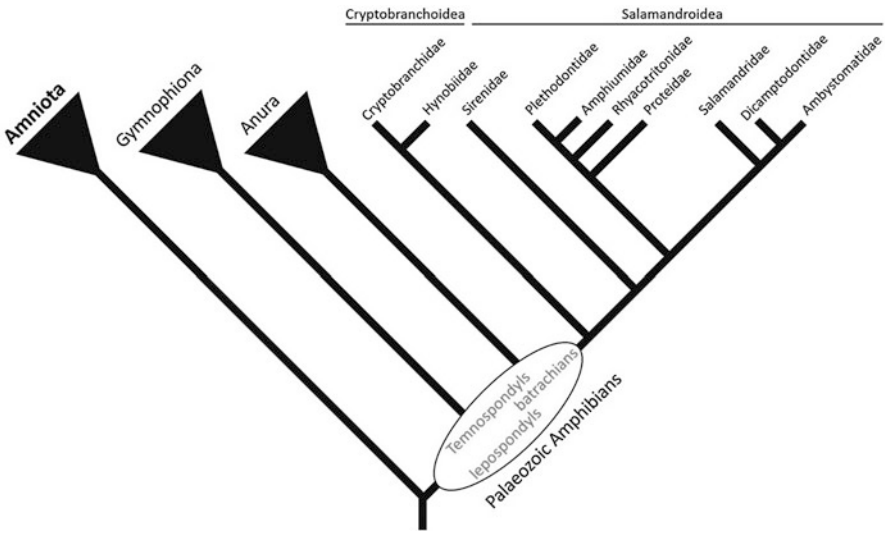


Fig. 2 Cladogram of relationships among extant amphibians, with the clade Amniota as the outgroup. The orders Gymnophiona and Anura are shown without expansion of their contained families. The order Urodela is expanded to show the relationships among its extant families, and the suborders they belong to are labeled. Due to uncertainty over how the extant orders are anciently related, a bubble containing orders of Palaeozoic Amphibians that have been argued to be the ancestors of some, or all, modern amphibians has been used as a basal node. Branch lengths do not represent any scale. (Modified from Shen et al. 2013)

Ambystomatidae known as the *Ambystoma tigrinum*, or tiger salamander, complex, a clade of approximately 17 closely related species spread throughout Mexico and parts of the United States (Shaffer 1993; Shaffer and Randal Voss 1996; Weisrock et al. 2006). The members of this species complex express low divergence levels, which together with analyses of glacial advances and retreats across the Great Plains has caused researchers to believe that some of the taxa within the complex are likely to be less than 18,000 years old (Shaffer 1993; Wade et al. 1994). The high genetic similarity amongst member species of this complex stands in sharp contrast to the relationships of the remaining Ambystomatidae species, whose observed genetic distances are an order of magnitude higher than the ones existing among species within the *A. tigrinum* complex (Shaffer 1993).

However, even though the members of the tiger salamander complex display high genetic similarity, especially within certain conserved regions such as alloenzymes and mtDNA (Shaffer 1984; Shaffer and Randal Voss 1996), significant physiological variation exists between many of the members. For example, a variety of species are neotenic, either partially or fully (Collins 1981; Shaffer 1993). These physiological characteristics initially led to the axolotl being grouped with the other neotenic species within the tiger salamander complex (Tihen 1958). However, later genetic analyses revealed that this grouping was almost certainly incorrect, with neoteny instead being gained and lost several times within the complex (Fig. 3) (Shaffer 1984). Therefore, the axolotl was placed as being more closely related to certain subspecies of *A. tigrinum*

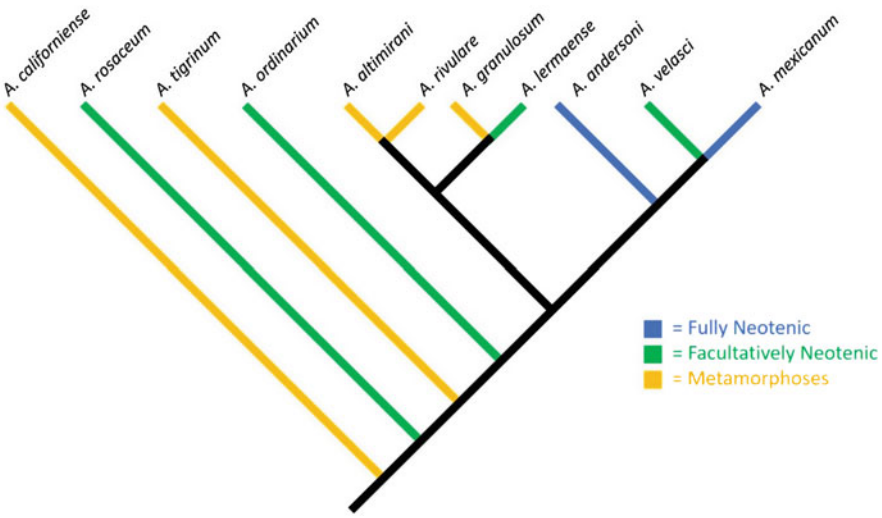


Fig. 3 Cladogram of some of the members of the tiger salamander species complex, showing current understandings of their relationships to each other. *Ambystoma californiense* is used as the outgroup of the species complex. Neoteny is indicated within the tree, and supports the general understanding that the trait has been gained and lost several times throughout the Ambystoma lineage. Branch lengths do not represent any scale. (Designed with data from Shaffer 1993; Shaffer and Randal Voss 1996; Williams et al. 2013; and O'Neill et al. 2013)

(Shaffer 1993), and was later updated again to being more similar to some subspecies of *A. velasci* (Fig. 3) (Shaffer and Randal Voss 1996). While this general positioning within the complex has been maintained by more recent studies that have used multiple nuclear loci (O'Neill et al. 2013; Williams et al. 2013), the exact relationships among species and subspecies within the tiger salamander complex will likely continue to be frustrated: specifically, by an overall genetic similarity extensive enough to allow certain species to interbreed with each other (Brandon 1972) and the potential elevation of subspecies to full species status, or vice versa.

Outside of the historical relationships of the axolotl to other salamander species, a much more recent issue may now play a major role on its development and evolution, namely the destruction of its natural habitat in Xochimilco by human urbanization. This has driven the population of axolotl in the wild to the brink of collapse (Contreras et al. 2009; Voss et al. 2015) and has resulted in them, along with several other species of Mexican salamanders, to be classified as critically endangered.

5 Limb Regeneration – The Limb as Multi-Tissue Structure

For decades, the salamander's seemingly endless regenerative abilities have attracted a lot of attention for its similarities with mammalian – but especially human – counterparts. Salamander limbs are complex structural and mechanical parts,

composed of tissues coming from all embryonic layers that are organized into functional, modular units (Payzin-Dogru and Whited 2018; Stocum 2017). The regeneration of muscle fibers, bone, connective tissue (CT), nerves, skin, and blood vessels back into an operational unit thus requires a tight spatial and temporal coordination of several events spanning many levels of complexity – from the cellular, to the tissue, and finally to the organ level –, which makes salamander limb regeneration an ideal model system for study. The promise of harnessing this potential for de novo formation of structures and using it to regenerate complete parts in mostly non-regenerative organisms, such as humans, has brought the study of salamander limb regeneration to the forefront of the field of regenerative medicine.

Limb regeneration in urodeles is a stepwise process that occurs in three main phases: wound healing, blastema formation, and growth and patterning (Bryant and Gardiner 2002; Payzin-Dogru and Whited 2018; Tank et al. 1976), and details of each will be explored in more detail in the rest of this chapter. After closure of the wound site through the formation of a specialized epithelium – the wound epithelium –, an array of somewhat undifferentiated cells, termed the blastema, accumulates underneath it and starts proliferating (Fig. 4b, c). After a growth phase, the seemingly homogeneous blastema begins to form the lost appendage through progenitor cell proliferation, differentiation, and directed outgrowth (Miller et al. 2019). However, although having been a source of fascination and study for over 200 years, since the work of Spallazani in the 1700s through to the age of genetics, the molecular details of regeneration are only now beginning to be unveiled.

5.1 Injury Signals

The very first events occurring after amputation are actually common to regenerative and non-regenerative injuries, in which the main objective is to restore tissue homeostasis and clear pathogens. Almost immediately, neighboring blood vessels will undergo vasoconstriction, followed by the rapid formation of a fibrin clot and the onset of an acute inflammatory response (Repesh and Oberpriller 1980; Sandoval-Guzmán and Currie 2018).

Even though the studies of injury signals and the inflammatory response in salamander limb regeneration are in their infancy, data from other regenerating contexts in other models have elucidated what are possibly shared mechanisms. Osmotic or necrotic cell swelling, calcium flashes, and gradients of reactive oxygen species (ROS) seem to be among the first "danger cues" triggered during wound healing and were shown to be able to recruit inflammatory cells (such as macrophages and leukocytes) to the injury site (Enyedi et al. 2016; Hervera et al. 2018; Niethammer et al. 2009; Razzell et al. 2013). Increased and sustained levels of ROS, in particular, were demonstrated to be important for blastema formation, post-injury directional axonal growth, and activation of canonical Wingless-related integration (Wnt) signaling and Fibroblast Growth Factor (Fgf) expression – all of which are key processes occurring in salamander limb regeneration (see below) (Hervera et al.

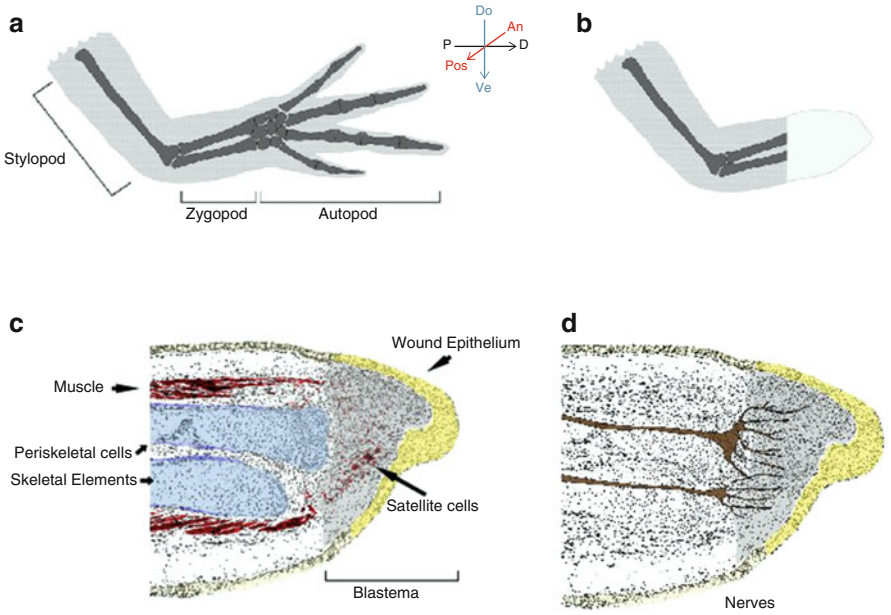


Fig. 4 (**a**) An axolotl limb with the three main sections, its 24 bones, and the different axis: posterior (Pos), anterior (An), dorsal (Do), ventral (Ve), proximal (P), distal (D). (**b**) After amputation at any level of the PD axis, a transient structure called the blastema will form, containing progenitor cells that will regrow the limb. (**c**) Cells retain memory of their origin when regenerating a limb. An inverse image of a 6-day blastema with nuclei stained with a nuclear marker (in black). In axolotl, muscle (in red) at the stump contributes to make new muscle through the muscle stem cells, called satellite cells (in red in the blastema). Skeletal elements (in blue) do not contribute cells to the blastema. Instead, periskeletal cells (in lilac) form the new elements along with further contribution of dermal cells. Epidermis in the stump (light yellow) will migrate to close the wound and form the wound epidermis (in bright yellow). (**d**) Axonal outgrowth into the blastema as thin fibers (in brown)

2018; Love et al. 2013; Simkin et al. 2017b) and have been recently implicated in axolotl tail regeneration (Al Haj Baddar et al. 2019).

An important component of this early response to injury is the migration of macrophages to the amputation site, which starts shortly after wound healing and lasts until the beginning of blastema formation (Godwin et al. 2013). Macrophages are known to clear dead cells, release pro-inflammatory cytokines, and later on produce molecules that dampen inflammation and stimulate angiogenesis, fibroblast migration, and cell division, in a dual role as professional phagocytes and local orchestrators of the immune response (reviewed in Park and Barbul 2004). In fact, systemic depletion of this cell population in salamander models of limb regeneration is enough to abrogate regeneration and induce fibrosis in the injury site, despite adequate wound closure and wound epithelium (WE) formation (Godwin et al. 2013); whereas in mouse digit tip regeneration they appear to be crucial both for WE formation and blastema outgrowth (Simkin et al. 2017b). Interestingly,

inflammation of the neighboring tissue after limb amputation in axolotl involves the simultaneous expression of both pro- and anti-inflammatory cytokines, which constitutes an interesting departure from mammalian models of injury in which the latter are only expressed long after the former (Simkin et al. 2017a). Indeed, a comparison of macrophage dynamics during ear-hole punch regeneration in mouse and the hyper-regenerative African spiny mouse (*Acomys cahirinus*) point to the existence of two macrophage subtypes, pro-inflammatory (M1) and repair (M2), that are spatially restricted to distinct tissue compartments and in which the former fail to infiltrate the regeneration blastema (Simkin et al. 2017a). Macrophages have also been implicated in the regulation of cytokine release from nonimmune system tissues, controlling the duration of the acute inflammatory response during zebrafish fin regeneration (Hasegawa et al. 2017). Conversely, early blastema cells were shown to produce IL8, an important pro-inflammatory factor that is able to recruit cells from the myeloid lineage and which promotes regeneration blastema formation and outgrowth (Tsai et al. 2019). Altogether, these results emphasize the importance of a robust and controlled inflammatory response in the first stages of tissue regeneration.

5.2 The Wound Epithelium (WE) and Formation of the Apical Epithelial Cap (AEC)

Following amputation and the initial damage control events at the injury site, the wound is rapidly covered by resident epidermal cells flanking its edges. These specific keratinocytes are rapidly mobilized and actively migrate over the lesion without proliferating, covering the exposed wound surface and forming the wound epithelium (WE) (Fig. 4c) (Ferris et al. 2010; Hay and Fischman 1961). In salamanders this process occurs in the span of hours (Carlson et al. 1998; Ferris et al. 2010; Repesh and Oberpriller 1980), which is 5–10 times faster than what is observed in mammalian cases of wound repair (Odland and Ross 1968; Yu et al. 2010). The WE is absolutely crucial for blastema formation, as illustrated by a variety of experiments using several – sometimes even creative – ways to abrogate its formation. Regeneration was inhibited when the wound epithelium was either periodically removed (Thornton 1957), had a flap of mature skin sutured on top of the amputation site (Mescher 1976), or had the amputated edge of the limb surgically implanted into the animal's body wall (Goss 1956b). However, if the lattermost procedure is sufficiently delayed, it will result in a blastema formation and subsequent limb regeneration inside of the body cavity (Goss 1956a).

After the rapid wound coverage, the cells of the WE will start proliferating, forming a multilayered structure designated as the apical epithelial cap (AEC) (Tank et al. 1976; Wallace 1981) (Fig. 4). Wnt signaling has been shown to be important for this formation, as inhibition of this pathway will specifically abrogate the proliferation of the WE, but not its migration (Kawakami et al. 2006), leading to defective regeneration. Similarly, inhibition of the Epidermal Growth Factor (EGF) cascade results in aberrant wound healing and a significant reduction in epidermal

cell proliferation, which implies an important role for it in the development of a functional WE/AEC (Farkas et al. 2016). Finally, transforming growth factor beta (TGF-β) signaling appears to be crucial to the process of wound closure and AEC formation during axolotl limb regeneration by acting through several mechanisms. Blockage of this pathway using specific inhibitors decreases overall proliferation in limb regenerates (Denis et al. 2016; Lévesque et al. 2007). Additionally, perturbations of TGF-β-dependent epithelial to mesenchymal transitions (EMTs), through combinatorial inhibition of both canonical (Smad2/3-dependent) and noncanonical (JNK- and p38-mediated) TGF-β signaling, were sufficient to delay wound closure and overall regeneration through the downregulation of several genes associated with this type of EMT (such as *Snail* and *N-cadherin*) in migrating keratinocytes (Sader et al. 2019).

The fact that interference with the formation of the WE and AEC has a decisive impact on the overall regenerative process illustrates their importance. In fact, gain-of-function experiments in which an extra WE/AEC was grafted onto an amputated limb stump resulted in the formation of extra limbs (Thornton 1957; Thornton 1960). The AEC, in particular, functions as a specialized epithelium known to secrete important molecules for the formation of the blastema, in such a way that one of its hallmarks is the absence of a basal membrane, which is thought to facilitate the diffusion of these factors (Neufeld and Aulthouse 1986). The AEC is itself an initiator of tissue remodeling, secreting metalloproteases (Mmps) – such as Mmp-2 and Mmp-9 – that break down the extracellular matrix, facilitating the mobilization of cells (Ferris et al. 2010; Lévesque et al. 2007; Yang and Byant 1994; Yang et al. 1999) while promoting blastema proliferation and maintenance through Fgf expression (Christensen et al. 2002; Han et al. 2001). Moreover, the WE expresses and releases regeneration-initiating extracellular factors upon injury that can specifically induce cell-cycle reentry in adjoining blastema cells, such as thrombin during newt limb regeneration and the axolotl MARCKs-like protein (AxMLP) during axolotl tail regeneration (Sugiura et al. 2016; Tanaka et al. 1999). The paradigm of tail regeneration, but this time in *Xenopus* larvae, was also used to find a population of epidermal cells, designated as regenerating-organizing cells (ROCs), that seem to be associated with regeneration-permissive states in this species. These cells were found to be scattered throughout the animal's epidermis, migrate specifically to the wound site to form the WE, and express important factors for regeneration such as Wnts, Fgfs, and TGF-βs (Aztekin et al. 2019). However, whether a similar population exists in axolotl remains to be discovered.

5.3 Blastema Formation and Outgrowth

Ultimately, factors secreted by the AEC will promote the accumulation of a transient mass of cells that will undergo proliferation, differentiation, and morphogenesis to regenerate the urodele limb (Fig. 4b, c) (Thornton 1960; Wallace 1981). Blastema cells start to appear 2 to 5 days post-amputation and are morphologically similar to the mesenchymal cells of the limb bud, having a small cytoplasm to nucleus ratio,

large numbers of free ribosomes, and a vesiculated endoplasmic reticulum, besides also possessing a great proliferative potential (Hay 1958; Hay and Fischman 1961; Salpeter and Singer 1960). After an initial phase of migration and accumulation under the AEC to form a stereotypical translucent conical structure (sometimes designated as the early bud blastema), blastema cells start proliferating and re-differentiating into new tissues (Stocum 2017) while the whole structure is progressively innervated and vascularized by new nerve fibers and capillaries that sprout from the severed ends of the ones found in the mature limb stump (Singer 1949; Singer and Inoue 1964). It is the combination of blastema cells and the WE, including the AEC, that will orchestrate the dynamics of limb regeneration (Muneoka and Sassoon 1992) (Fig. 4).

5.3.1 Origin of the Blastema Cells

Formation of a blastema is, thus, absolutely crucial for proper limb regeneration. This has been demonstrated through irradiation experiments during the first half of the twentieth century, whereby x-ray-treated limbs were unable to regenerate due to the loss of this structure (Butler 1933; Butler and O'Brien 1942). The same experiments also demonstrated that, unlike planarians, blastema cells in salamanders originate locally (Butler and O'Brien 1942), with widespread cellular proliferation occurring approximately 1 mm proximal to the site of amputation (Hay and Fischman 1961). However, the nature and definitive origin of blastema cells has long been debated. The classical definitions described the blastema as a mass of pluripotent cells that would contribute to all regenerated structures, in a similar fashion to stem cells. The fact that successive re-amputations of the same limb generally result in reliable regeneration of the structure seemed to lend some strength to this hypothesis (Bryant and Gardiner 2002; 2017a). However, recent experiments using grafting or lineage tracing approaches have proved that blastema cells – homogeneous and undifferentiated as they may appear – are actually a collection of semi-fate-restricted progenitor cells resulting from either a mobilization of resident tissue-specific stem cells or from a process of cell dedifferentiation (Currie et al. 2016; Kragl et al. 2009; Sandoval-Guzmán et al. 2014). The specific origin of each tissue in the regenerate will be discussed at length later on in this chapter.

5.3.2 Requirements for Blastema Formation and Proliferation

Several factors are known to influence blastema formation and proliferation, but the more important ones seem to be the initial tissue histolysis after amputation, innervation, and contact between cells from the anterior and posterior sides of the limb. Together, these processes will contribute to the transition from a normal wound healing to a full regeneration response.

Histolysis

Tissue histolysis, or the local degradation of extracellular matrix (ECM) in the vicinity of the amputation surface, is an important event contributing to blastema formation and is thought to be one of the earliest signals triggering blastema cell

migration and dedifferentiation (Sandoval-Guzmán and Currie 2018). Shortly after amputation, several types of proteolytic enzymes – particularly lysosomal acid hydrolases and Mmps – are secreted into the mature tissues mainly by macrophages and cells from the WE/AEC (Vinarsky et al. 2005; Yang and Byant 1994; Yang et al. 1999). Histolysis lasts until the onset of blastema differentiation and seems to be mostly controlled through the activity of tissue inhibitors of metalloproteinases (TIMPS), which are upregulated when Mmp levels reach maximum levels in the stump tissues (Stevenson et al. 2006). The importance of tissue histolysis in regeneration was demonstrated through the pharmacological inhibition of MMP activity, which resulted in defective regeneration and tissue fibrosis (Vinarsky et al. 2005). In fact, ECM degradation releases fibroblasts from the dermis, interstitial connective tissue of muscle, periosteum, and nerve sheath, as well as Schwann cells from the peripheral nerves (Stocum 2017), all of which will make up the cellular compartment of the blastema and later contribute progeny to all the new tissues of the limb regenerate. Additionally, data from other regenerating contexts seem to indicate that the degraded matrix, by having unique material properties such as a lower rigidity, can provide instructive signals able to direct dedifferentiation processes and is likely to create durotactic cues directing migration toward the injury site (Chen et al. 2016; Godwin et al. 2014; Yahalom-Ronen et al. 2015). Additionally, the newly deposited blastema-associated ECM is typically composed of high levels of fibronectin and glycosaminoglycans (GAGs) which are known to support high rates of proliferation, robust cell migration, and promote later progenitor differentiation (reviewed in Godwin et al. 2014).

Nerve Dependence

The link between innervation and regeneration has been known since the very early days of experimental work on limb regeneration, back in the mid-nineteenth century. Surgical denervation of salamander limbs before amputation or in the early stages of regeneration completely abrogated the process (Schotté and Butler 1941; Singer 1946) and later studies revealed that this failure was due to the inability to develop a mature blastema; instead, a stump is formed containing a few blastemal cells with defective cell cycles underneath a cap of fibrotic scar tissue (Goldhamer and Tassava 1987; Liversage and McLaughlin 1983; Maden 1978; Singer and Craven 1948). Yet, denervation at later stages of the regenerative process (later than 13 days post-amputation) results in perfectly specified regenerates, although generally smaller (Schotté and Butler 1944; Singer and Craven 1948), which implies different effects of innervation at specific stages of regeneration. Notably, in axolotl, once the limb has been reinnervated, regeneration proceeds normally following a second amputation (Schotté and Butler 1941); however, there seem to be important differences among salamander species, as newts are incapable of reinnervating the limb and, thus, are permanently unable to regenerate after a single denervation event (Liversage and McLaughlin 1983).

Interestingly, not only does there seem to be a minimum amount of nerves necessary for a successful regeneration response, but also all nerve fibers – either sensory or motor – contribute roughly equally to the onset of regeneration (Singer

1946). Conversely, deviating a nerve to a non-regenerating wound in the epidermis will, under certain conditions, generate a fully functional blastema capable of producing an ectopic limb, in a technique called the Accessory Limb Model (ALM) (Endo et al. 2004; Satoh et al. 2007). These findings led to the hypothesis that peripheral nerves produce a threshold level of key factors necessary for blastema formation and growth, also known as the neurotrophic hypothesis of nerve support. Since then, several factors have been implicated in nerve-dependent regeneration and, specifically, as having an effect on blastema cell proliferation under these conditions (reviewed in Farkas and Monaghan 2017), such as substance P (Globus et al. 1991), insulin (Liversage et al. 1984), transferrin (Kiffmeyer et al. 1991; Mescher et al. 1997), and Nerve Growth Factor (NGF) (Weis and Weis 1970). Some molecules, in particular, can completely rescue regeneration in denervated limbs. In newts, newt Anterior Gradient protein (nAG) is expressed in Schwann cells along the nerve sheath and in the AEC, and becomes downregulated upon denervation. Overexpression of nAG through electroporation in nerve-deficient limbs is enough to induce blastema formation in an otherwise nonpermissive condition, and in vitro studies have shown that nAG effects are mediated by its receptor in blastema cells, Prod1 (Kumar et al. 2007). Similarly, supplying Neuroregulin-1 (Nrg1) to a denervated newt blastema induces regenerative growth by increasing cell proliferation (Farkas et al. 2016). The use of the ALM opened up new ways of analyzing the exact role of several molecular factors and pathways responsible for nerve-dependent regeneration. Fgfs and bone morphogenetic proteins (BMPs), for example, were found to be expressed in the nerves sprouting from the dorsal root ganglia and are downregulated upon denervation (Mescher and Gospodarowicz 1979; Mullen et al. 1996). Co-application of Fgf8 and Gdf5 can induce accessory limb formation upon lateral wounding in the absence of a deviated nerve by sustaining blastema cell proliferation (Makanae et al. 2013, 2014; Mescher and Gospodarowicz 1979), whereas downregulation of *Fgf8* and *Bmp7* specifically in dorsal root ganglia delayed, or even entirely abrogated, limb regeneration upon amputation (Satoh et al. 2016). Remarkably, in some instances a limb can regenerate without innervation. This is the case in aneurogenic limbs, whereby limb regeneration will indeed occur without a nerve supply provided that the limb itself has developed without one (specifically by removing the neural tube at the level of the forelimb during the early embryonic stages) (Yntema 1959). Nerveless limbs grafted onto innervated animals retained their ability to regenerate; however, these limbs irreversibly lost their nerve-independence as soon as innervation by the host nerves was complete (Steen and Thornton 1963). Yet, it was found that some nerve-independence could be regained by extensive periods of denervation (Thornton and Thornton 1970). This apparent "addiction" of limbs to nerves has been hypothesized to be due to the reliance on regeneration-critical factors which are first produced by some other structure, and are then later supplied exclusively by the nerves upon innervation. One possibility is that the AEC is the structure that initially provides the diffusible molecules necessary for blastema proliferation, but that its expression is later downregulated and the role is taken over by the nerve once it reaches the most distal part of the regenerate. Without a nerve present, there is no structure left to produce the necessary signaling

molecules, and these essential factors putatively produced by the AEC would wane to levels insufficient for blastema growth (Stocum 2017).

The role of the nerve supply is not restricted to directly promoting blastema proliferation, but also acts in the formation of the WE and AEC. The latter is extensively invaded by nerves during the first stages of regeneration (Singer 1949), and reinnervation of the AEC is coincident with a ten-fold increase in proliferation of blastema cells (Loyd and Tassava 1980; Mescher and Tassava 1975). In fact, early innervation seems to have a key role in the transition of WE into a secretory AEC (Satoh et al. 2012), appearing to be the main factor distinguishing a proper functional AEC from the neoepidermis that forms atop non-blastema-based regenerating skin wounds (Endo et al. 2004; Satoh et al. 2008). Specifically, innervation promotes expression of *Sp9* and *Dlx3* in the basal layers of the AEC, two genes implicated in embryonic limb bud development and as targets of Fgf signaling (Mullen et al. 1996; Satoh et al. 2008). Besides its effect on cell proliferation and supply of mitogenic factors, denervation also drastically reduces transcription and protein synthesis in the stump tissues, specifically of ECM components such as proteoglycan and collagen-associated glycosaminoglycans (Mescher and Munaim 1986; Monaghan et al. 2009) which, as stated above, are important for a competent regenerative response.

Notably, the relationship between regenerating nerve fibers and the blastema cells is a reciprocal one, as the latter are known to produce factors that promote axon growth and invasion (Richmond and Pollack 1983). In fact, blastema cells can influence the transcriptome of dorsal root ganglia (DRGs) as comparison studies have shown that DRG genes were differentially expressed in the presence of blastema cells (Athippozhy et al. 2014).

Anterior-Posterior Tissue Interactions

However, neither tissue histolysis nor innervation alone is sufficient to sustain the necessary blastemal proliferation for complete regeneration. In fact, deviation of a nerve in the ALM model will only successfully form an accessory limb if a piece of tissue from the opposite side of the injury is simultaneously grafted next to the wound (Endo et al. 2004; Satoh et al. 2007). The importance of this contact between anterior and posterior tissues is underlined by the observation that transplantation of a left limb blastema onto a right limb stump (or vice versa) yielded regeneration of three limbs, the normal one and two other supernumerary accessory limbs (Iten and Bryant 1975; Maden 1980; Stocum 1982).

The molecular basis of this phenomenon in axolotl was found to be a feedback loop between Sonic Hedgehog (*Shh*) and Fgf signaling occurring in blastemal cells (Nacu et al. 2016). Blastemas composed solely of anterior tissue regress rapidly, yet activation of hedgehog signaling was sufficient to drive complete regeneration through the activation of the Fgf pathway. Conversely, blastemas entirely formed by posterior tissues were able to properly grow and regenerate a complete limb only when supplied with Fgf8. These findings demonstrate that there are important complementary cross-inductive signals produced by anterior and posterior tissues that are required for blastema outgrowth, and that juxtaposition of anterior and

posterior signals plus innervation is necessary and sufficient for proper limb regeneration.

5.3.3 Relevant Molecular Factors Necessary for Blastema Formation and Proliferation

Fgf signaling is one of the major pathways involved in blastema proliferation. *Fgf8* is expressed in the deep layers of the anterior AEC and blastema cells directly underneath it, and it is necessary to maintain blastema proliferation and the expression of other blastema-associated genes (Albert et al. 1987; Chew and Cameron 1983; Han et al. 2001). Maintenance of *Fgf8* expression during regeneration depends not only on *Shh* and hedgehog signaling, but also seems to be influenced by the canonical Wnt pathway (Kawakami et al. 2006; Nacu et al. 2016; Yokoyama et al. 2007). So far, the most important receptor for the Fgf pathway during regeneration seems to be FgfR2, which is also extensively present in blastema cells (Poulin and Chiu 1995; Poulin et al. 1993). Other factors associated with proliferative ability, such as Nrg1 receptors Erbb2 and Erbb3, as well as nAG receptor Prod1, are likewise found in a significant proportion of basal AEC and blastemal cells, and possibly link blastemal proliferation to nerve dependency (Farkas et al. 2016; Kumar et al. 2007). RNA binding proteins were found to be involved as regulators of continuous blastema proliferation as well. *Piwi-like proteins 1* and *2* (*PL1* and *PL2*) and *Cold-induced RNA binding protein* (*Cirbp*) are expressed in blastema cells, and their depletion halts blastema growth and increases cell death (Bryant et al. 2017b; Zhu et al. 2012). Similarly, knockdown of *Kazal-type serine peptidase inhibitor domain 1* (*Kazald1*) abrogates blastema growth and results in a significant delay of chondrification and digit differentiation.

Interestingly, several stemness-associated genes, such as *Octamer-Binding Transcription Factor 4 (Oct4)*, *Sex Determining Region Y Box 2 (Sox2)*, and *Lin28*, were shown to be expressed during regeneration of the newt limb despite the fact that blastema cells are not pluripotent (Maki et al. 2009; Rao et al. 2009).

Finally, *Prrx1* is considered to be an important blastema marker and has been used in a variety of lineage tracing studies in regenerating structures (Currie et al. 2019; Gerber et al. 2018). This paired homeobox protein acts as a transcription factor and is activated in dermal fibroblasts contributing to blastema formation during axolotl limb regeneration (Satoh et al. 2007; Satoh et al. 2011).

5.4 Blastema Differentiation and Patterning

Once the blastema has proliferated enough to reach a critical size, it flattens and starts undergoing patterning processes to give rise to all the structures in the new limb. This involves the differentiation or redifferentiation of blastema cells into all of the necessary cell types at the right positions, while also becoming continuous and properly integrated with the mature tissue. One of the first events during this redifferentiation phase is the establishment of appropriate innervation and vascularization in order for the new tissue to survive (Smith and Wolpert 1975). The second

key process is the establishment of the main limb axes: the proximo-distal (PD), antero-posterior (AP), and dorso-ventral (DV) axes (Fig. 4a, b).

Transplantation experiments throughout the years have revealed that while early blastemas either fail to develop or develop according to the host site when grafted ectopically (e.g., limb blastema grafted onto a tail amputation site becomes a tail regenerate and vice versa), older blastemas develop according to the tissue of origin. Strikingly, older blastemas also preserve their polarity, which means that if transplanted with their distal end facing the proximal stump tissue, they will regenerate distal structures (e.g. structures such as fingers) in proximal positions (middle of the arm) (reviewed in Stocum 1984). Combined, these experiments have shown that, once it reaches a certain size, the blastema is relatively autonomous and that both the blastema identity (limb blastema vs. tail blastema, for example) and patterning are determined shortly after its proliferative stage (Stocum 2017).

5.4.1 Proximo-Distal (PD) Axis

Grafting experiments using blastemas from different PD amputation levels have demonstrated that urodele limb regeneration follows the rule of distal transformation, whereby only the missing structures distal to the amputation plane will regenerate, irrespective of the amputation position within the limb. As such, if a hand blastema is transplanted onto the stylopod (upper arm) amputation site, a full limb will regenerate, but the donor blastema cells will only contribute to the distal-most structures. Strikingly, even when the limb is forced to regenerate with a reversed polarity, as in the case of the circular limb model – in which amputation in the zeugopod (lower arm) is followed by implantation of that extremity in the body cavity of the animal, after which the limb is subsequently re-amputated at the level of the stylopod – the repositioned arm will regenerate all of the structures distal to the stylopod (Nacu and Tanaka 2011).

The main signaling cascade controlling PD axis specification seems to be retinoic acid (RA). Exogenous supplementation of regenerating limbs with RA leads to a duplication of proximal elements in a dose-dependent manner (Maden 1983), whereas blastemas initially originating from distal amputations can be instructed to give rise to proximal structures if treated with RA (Crawford and Stocum 1988). Activation of RA signaling during limb regeneration is observed in the AEC and the peripheral nerve fibers and their associated cells (Monaghan and Maden 2012; Scadding and Maden 1994). However, upon exogenous RA administration, a proportion of blastema cells exhibit activation of this signaling cascade, which indicates that these cells are capable of transcriptionally responding to RA even if they typically do not (Monaghan and Maden 2012).

Several factors have been discovered to mediate RA's effect in PD axis determination and, particularly, on its proximalizing activity. The newt-specific protein Prod1 was one of the first to be characterized. RA supplementation induces overexpression of Prod1 in blastemas, particularly those formed upon proximal amputations and in the proximal-most regions of the mature limb (Da Silva et al. 2002). Importantly, overexpression of Prod1 pushes distal blastema cells to translocate to more proximal locations and assume proximal identities

(Echeverri and Tanaka 2005). Similarly, overexpression of *Meis1* (*Myeloid Ecotropic Viral Integration Site 1*) in blastemas led to proximal localization of expressing cells in the regenerate limb, whereas *Meis* knockdown inhibits RA proximalizing activity (Mercader et al. 2005).

PD cues seem to be relayed specifically by CT-derived blastema cells which, besides expressing these RA signaling downstream effectors, express genes that are also involved in PD specification in the embryonic limb, such as *HoxA9* and *HoxA13* (Kragl et al. 2009; Nacu et al. 2013) (see Sect. 6).

5.4.2 Antero-Posterior (AP) Axis

Similar to embryonic limb development, AP axis specification in the regenerating limb relies on *Shh* signaling, which is expressed in the posterior side of blastemas. Forced expression of *Shh* in the anterior side causes ectopic digit formation (Roy et al. 2000). Conversely, treatment with the Shh inhibitor cyclophosphamide abrogates posterior digit formation (Roy and Gardiner 2002).

5.4.3 Dorso-Ventral (DV) Axis

Little is known about the mechanistic details of DV axis specification during urodele limb regeneration. However, in *Xenopus* tadpole blastemas, there are indications that, similar to AP axis specification, the genes used in the limb for DV specification during embryonic development are likewise expressed in regenerating ones (Matsuda et al. 2001) (see Sect. 6).

5.5 The Source of Cellular Diversity – From the Blastema to the New Tissue

After amputation, all tissues of a functional limb are regenerated, which include the epidermis, dermis, muscle, soft connective tissue, and cartilage and bone (Fig. 4c,d). As such, blastema cells must proliferate and differentiate in a coordinated manner in order to substitute the missing structure and restore its functionality, in addition to contributing to the reestablishment of innervation and of an adequate blood supply. Once thought to be a mass of pluripotent cells able to generate all the necessary tissue types, blastema cells were found to be, instead, a heterogeneous collection of lineage-restricted progenitors deriving from the differentiated mature tissues of the limb stump. Specifically, most blastema cells originate from connective tissue (particularly dermal fibroblasts), myogenic cells, and Schwann cells (Kragl et al. 2009) (Fig. 4).

5.5.1 The Connective Tissue (CT)

CT cells encompass bone and cartilage, tendons, periskeleton, and dermal and interstitial fibroblasts. The majority of blastema cells originate from various sources of CT in the mature limb, particularly from dermal fibroblasts (Dunis and Namenwirth 1977; Kragl et al. 2009; Muneoka et al. 1986). These CT cells undergo a process of dedifferentiation that is able to revert them to a relatively homogeneous progenitor cell state similar to the one found in the embryonic limb bud – including

the ability to differentiate into various cell types within the connective tissue lineage (Gerber et al. 2018). This multipotent state subsequently results in an extensive contribution from CT cells not only to dermis, tendons, and interstitial fibroblasts, but also to other structures, such as the formation of new cartilage (Kragl et al. 2009). Although chondrocytes do proliferate in response to amputation, these cells do not contribute to the blastema in axolotl digit tip regeneration; instead new cartilage is generated from transdifferentiated dermal fibroblasts and periskeletal cells (Currie et al. 2016). Interestingly, the same study showed that the time at which CT cells entered the blastema corresponded to whether it was likely to form a skeletal (cartilage) cell or a soft connective tissue cell: early-migrating cells seemed to contribute to regenerated cartilage, whereas late-migrating ones generated essentially soft connective tissue in lateral regions of the regenerate (Currie et al. 2016).

5.5.2 Muscle

However, not all cell lineages found in the new limb go strictly through a process of dedifferentiation, and not all salamander species regenerate in the same way. In axolotl adults and in newt larvae, for example, new muscle arises from resident muscle stem cells – the satellite cells - that are released and subsequently proliferate in the limb blastema (Fig. 4c) (Kragl et al. 2009; Sandoval-Guzmán et al. 2014). In contrast, in adult newts the main contribution to regenerated muscle comes from myofiber dedifferentiation; limb amputation induces the fragmentation of myofibers into mononucleated cells, which will proliferate and give rise to the skeletal muscle in the new limb (Sandoval-Guzmán et al. 2014). Muscle regeneration is, thus, a remarkable case in which the mechanisms and the origin of the cells vary according to both developmental stage and species.

5.5.3 Schwann Cells and Other Nerve-Associated Mesenchymal Cells

Early experiments using nerves grafted into irradiated hosts suggested that Schwann cells – nerve associated cells that myelinate and support axons (Frostick et al. 1998) – were able to dedifferentiate into diverse cell types during limb regeneration (Wallace 1972). Modern cell tracing techniques, however, have demonstrated that, even though they seem to dedifferentiate, and even migrate to the blastema in axolotl limb and mouse digit tip regeneration, these cells will strictly give rise to new Schwann cells in the nerve tract of the new regenerate (Johnston et al. 2016; Kragl et al. 2009). Nevertheless, Schwann cells are thought to be important for nerve regeneration via the production of factors that promote axonal growth and guidance into the new tissues (Frostick et al. 1998; Johnston et al. 2016; Stocum 2017). Yet, in murine models of digit tip amputation, some nerve-associated mesenchymal cells in the endoneurial compartment of the nerve sheath were shown to contribute significantly to bone regeneration and dermal repair (Carr et al. 2019).

5.6 Growth Control

Not much is known about the molecular mechanisms used to stop regeneration and, especially, the way the overall tissue detects the final dimensions of the structure so

that the regenerated limb can reach a satisfactory size and acquire the adequate proportions to be successfully integrated into the mature organism. However, denervating the regenerate after the first stages of blastema formation will result in a miniaturized limb (Bryant et al. 2017a; Singer and Craven 1948). This striking effect on the dimensions, but not on the patterning, of the regenerate implicates a role of the nerve supply in blastema proliferation and, perhaps, also on the growth phase following patterning.

5.7 Final Considerations

There are still many unanswered questions regarding the molecular players and signaling pathways involved in the various stages of regeneration, how these factors interact with each other, and how similar and different the regeneration process is in the various model organisms. However, the recent explosion of high-throughput genome wide sequencing studies – both at whole tissue and at single cell resolution – have highlighted the complexity and highly dynamic nature of the blastema transcriptome (Bryant et al. 2017b; Gerber et al. 2018; Leigh et al. 2018) throughout the regenerative process, which will likely open up many research avenues in the future.

6 Development and Regeneration

One of the most prevalent misconceptions about regeneration is that it is simply a recapitulation of development. However, if regeneration is merely reusing developmental pathways, then the obvious question is why do some animals regenerate while others do not, since most of them undergo embryonic development. In fact, a growing body of data has revealed that the link between development and regeneration is much more complex and nuanced (Nacu and Tanaka 2011; Tanaka 2016). Even though much of the genetic toolbox that is used is common to the two processes, and that both require a tight spatial and temporal coordination of several events spanning many levels of complexity, regeneration has additional layers of intricacy since its start and end points are seldom fixed. In the particular case of the salamander limb, unlike development, regeneration can occur during the entire lifetime of the animal and is not restricted to only one location along the limb PD axis. As the ultimate goal of regeneration is to restore the function of a structure that is part of a fully formed organism, mature cells along the entire limb need to have a remarkable degree of plasticity and inherent positional information cues for long periods of time so that not only are the proper missing tissues replaced, but that they are also adequately incorporated into the mature tissue.

6.1 *"If it ain't broke, repurpose it."*: Common Factors and Molecular Pathways Used in Development and Regeneration

Regeneration is thought to be one example of co-option of developmental circuits into new contexts, many of them with broadly the same function. The limb, in particular, is a fertile ground for comparisons since most of the underlying mechanisms involved in its formation and patterning are well-known.

Early embryonic limb buds and regenerating limb blastemas, albeit very simple, share important structural similarities. Particularly, both consist of a mass of mesenchymal cells encompassed, respectively, by an ectodermal epithelium or epidermis, which will eventually develop a distal epithelial thickening that is absolutely necessary for limb growth (Gilbert 1997; Thornton 1957). This structure is designated as the apical epithelial cap (AEC) in the regenerating limb and as the apical ectodermal ridge (AER) in the developing limb bud, which are considered to be structurally and functionally analogous given their similar roles as distal signaling centers (Stocum 2017). Importantly, both structures secrete Fgf8, which has been shown to keep cells in an undifferentiated state and promote cell proliferation and survival (Chew and Cameron 1983; Han et al. 2001; Nacu et al. 2016).

Additionally, limb regenerates re-specify most of their axes using the same general mechanisms as in limb development. As previously mentioned, the *Shh* pathway has a conserved role in the establishment of the AP axis through its expression in the posterior region of the regenerating tissue, which is akin to the zone of polarizing activity (ZPA) observed in early stages of the embryonic limb (Nacu et al. 2016; Roy and Gardiner 2002). Likewise, some studies in *Xenopus* tadpole regeneration suggest that LIM Homeobox Transcription Factor 1 Alpha (Lmx1a) secreted from the dorsal mesenchyme might contribute to the specification of the DV axis in the blastema, opening the possibility that the rest of the molecular cascade – which involves *Wnt7a* and *Engrailed homeobox 1* (*En1*), activating and repressing the dorsal fate, respectively – is also acting during limb regeneration (Matsuda et al. 2001). However, the most striking case is the repatterning of the limb PD axis. CT-derived blastema cells have been shown to convey the proper positional information during axolotl limb regeneration and express several factors associated with PD axis determination, particularly *Hox* genes (Nacu et al. 2013). As in limb development and embryonic axial extension, *Hox* gene expression in regenerating limbs displays spatial and temporal collinearity: *Hoxa9* is expressed first throughout the blastema, followed by *Hoxa11* in the prospective lower arm region, and, finally, by *Hoxa13* in the prospective hand region (Roensch et al. 2013). *Meis1* and *Meis2* are two other genes expressed preferentially in the prospective upper arm region, and both have a role in RA-dependent proximal specification during limb development and regeneration (Mercader et al. 2005; Pownall and Isaacs 2010). Indeed, several observations have implicated a combinatorial effect of *Hoxa9/Meis1/2* in the establishment of the PD pattern in the stylopod (upper arm), *Hoxa9/11 /Meis2* in the zeugopod (lower arm), and *Hoxa9/13* in the autopod (hand region) (Mercader et al. 2005; Roensch et al. 2013) (Fig. 4a).

Finally, the dedifferentiation process occurring in CT cells to form the blastema will likely require similar changes in transcriptomic signatures and corresponding epigenetic landscapes to the ones observed during the reprogramming of cells to more stem-like states, such as in the generation of induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). Interestingly, gene members of the core pluripotency network are observed in newt blastema cells (Maki et al. 2009; Osorno et al. 2012; Rao et al. 2009). Despite not being enough to revert cells back to a pluripotent state, these factors might be essential for the correct reprogramming of CT cells to the embryonic-like stage that was recently reported to occur during axolotl limb regeneration (Gerber et al. 2018). Likewise, the reported expression and importance of *Piwi-like proteins 1* and *2* in blastema cells, which are involved in germline development and self-renewal (Zhu et al. 2012), highlights the usage of the same molecular players in the maintenance of a stem-like state in blastema cells.

6.2 *"But if you can't repurpose it…"*: Key Differences Between Regeneration and Development

However, despite all the commonalities, the main difference between the development and regeneration of complex structures is the fact that cells follow essentially opposite developmental trajectories. Development starts with only one cell, which will proliferate and generate progeny that will gradually differentiate, first into the major germ layers – the outermost ectoderm, the middle mesoderm, and the innermost endoderm –, and then further on into the terminally differentiated functional cell types that make up each particular tissue (Wolpert et al. 2002). Additionally, the limb bud mesenchyme during the first stages of development is composed of cells that are relatively equivalent and, thus, will have the same potency to give rise to all mature limb tissues (Gilbert 1997). Although some organisms like the planaria use resident pluripotent cells to form a blastema and regenerate an entire body (Knapp and Tanaka 2012), urodele and mammalian instances of regeneration rely instead mostly on mature cells, which is by itself a challenge. In axolotl limb regeneration the blastema is composed of a heterogeneous population of cells, and the CT cells comprising the majority of the blastema must first undergo a process of dedifferentiation whereby they acquire an embryonic limb bud-like identity at the transcriptional level before proliferating to generate the necessary cell numbers to rebuild the missing structure (Gerber et al. 2018).

Regeneration and development also occur at very different temporal and spatial scales, sometimes several orders of magnitude apart. Depending on the organism, limb development can take days or weeks to occur. Axolotls, for instance, require approximately 6 weeks until the forelimb is fully developed (Nye et al. 2003); on the other hand, limb regeneration progresses fairly quickly, and all of the main components of the limb are mostly restored just 3 weeks after amputation (Tank et al. 1976). This suggests that dedifferentiation, but especially proliferation, redifferentiation, and repatterning of cells in the blastema proceed at a greater pace than the seemingly equivalent processes of differentiation and patterning during limb development. The

problem of scaling and spatial distances is also a lot more taxing in regenerating structures. Limb morphogenesis during development predominantly relies on molecular gradients that affect cells that are only micrometers apart (Gilbert 1997; Wolpert et al. 2002). However, and particularly for the limb, the regeneration process has to substitute structures whose final size can span several millimeters in all three axes, and integrate them into the mature tissue. This leads to another interesting challenge uniquely faced by the mechanisms of regeneration, which is growth control and determination of the final size of the regenerate. For the missing part to be appropriately sized so that it can function properly in the context of the animal's proportions, there must be a sensing mechanism – either local or systemic – that can sustain fast blastemal proliferation for relatively short periods of time, while arresting tissue growth once the structure's final dimensions are achieved.

Finally, a key aspect of regeneration is the integration of the newly regenerated tissue with the preexisting mature one. This stands in sharp contrast with embryonic development, in which a given structure is mostly built stepwise from a relatively pluripotent cell population through a gradual process of differentiation. Yet, integration of the new and old tissue is extremely important given that it will have a decisive role in both the functional and structural integrity of the regenerated organ or structure. An additional complication to this problem is the fact that regeneration can occur after amputations at various positions in the limb, which means that repatterning and positional memory have to be tightly linked so that the end result is integrated and fully functional in the context of a wholly mature organism.

Although more and more studies have pointed out that, for regeneration to occur this fast and accurately, cells at the amputation site must already contain most of the necessary positional information and extraordinary proliferative abilities, not much is known about the particular mechanisms involved either in ensuring the adequate structural scaling or in how tissue is integrated.

6.3 New Technologies to Study Limb Regeneration

For many years, the field of regeneration was limited by the relative lack of modern genetic tools, which mostly stemmed from the fact that the great majority of master regenerators are not traditional model organisms. However, the advancement of technology and the dawn of high-throughput sequencing have made these organisms more amenable for the use of these tools.

An important step in the field of regeneration was the complete sequencing of the hydra, planaria, and axolotl genomes (Chapman et al. 2010; Grohme et al. 2018; Nowoshilow et al. 2018). This provided an important groundwork for the adaptation of most of the genetic tools that were previously established in more conventional model organisms. Another crucial technological development was the remarkable advancements in microscopy and live imaging techniques. This, coupled with the usage of fluorescent reporter lines and the fact that most model organisms for regeneration are relatively small and transparent, allowed for an unprecedented look into the regenerative processes at a cellular level. Light sheet microscopy, in

particular, allows for prolonged imaging with minimal interference of physiological processes that occur during either regeneration or development (Marques et al. 2019). Together, these new methodologies prompted a renewed interest in the study of regeneration: not only is it now possible to characterize the regenerative process at the molecular, genetic, and cellular levels, but also the acquired knowledge on how regeneration takes place naturally in such a great diversity of animals can provide very important insights into the field of regenerative medicine. The following table summarizes the variety of tools and techniques used to study regeneration (Table 1).

7 Mammalian Regeneration

Mammals are among the organisms that demonstrate a limited capacity for regeneration. The fact that this ability is temporally and locally restricted has encouraged the idea that mammals lack any ability to regenerate, even though there are plenty of regenerative examples of cellular plasticity that rescue the morphology and function of different parts of the body.

Cardiac regeneration is possible in mouse until a few days after birth (Porrello et al. 2011). This short window of time is, nevertheless, a big window of hope to understand the potential of mammals to regenerate. Indeed, after partially resecting a heart, new cardiomyocytes will arise from preexisting ones, which will then restore the heart's morphology and function. However, 7 days after birth, cardiomyocytes exit the cell cycle and the response to injury becomes fibrotic. Liver regeneration, on the other hand, is carefully referred to as compensatory regeneration, since the tissue is restored via hypertrophy of existing cells. In mouse, partial hepatectomy of up to two-thirds of the total size of the liver is the most common paradigm used to study liver regeneration. Upon surgical resection, direct recruitment and proliferation of differentiated cells (hepatocytes) in the remaining liver is able to replace the lost tissue in 7 to 10 days (Miyajima et al. 2014), without the apparent participation of tissue-specific stem cells (Miyaoka et al. 2012). However, the liver is also regenerated when the damaged is caused by other pathological conditions, such as viral infections or toxins. In these cases, the cellular response includes the proliferation of an epithelial cell population with intermediate phenotypes between hepatocytes and cholangiocytes (epithelial cells lining the bile ducts), referred to as "liver progenitor cells" (Fausto 2004; Roskams et al. 2003). This cell type is also injury-responsive when the proliferation of hepatocytes is compromised.

The intestine is a tissue that is constantly renewed by cells located at the base of the intestinal crypts, which move along their differentiation path from the crypts into the villus tip, ultimately dying 4–5 days later. These same cells, identified by a Wnt target gene, *Lgr5* (leucine-rich repeat-containing G-protein coupled receptor 5), will proliferate in response to injury (Takashima et al. 2013). Upon depletion of $Lgr5^{+}$ cells, a reserve stem cell identified as +4 replaces them (Tetteh et al. 2016; van Es et al. 2012). Furthermore, the loss of crypt stem cells promotes dedifferentiation of adjacent cells to reacquire a stem cell potential (van Es et al. 2012).

Table 1 Summary of tools and techniques used to study regeneration. Based on Marques et al. 2019

	Method	Application	Advantages	Disadvantages
Genome manipulation tools	Fluorescent reporter lines	Cell/tissue imaging	• Assessment of gene expression by promoter/enhancer driven fluorescent reporter expression	• Detection limited to promoter activity • Regulatory region might not recapitulate fully the endogenous gene expression pattern • Fluorescent protein expression might be more stable than the endogenous gene
	Photoconvertible proteins	Lineage tracing	• Activated by light	• Transient labelling • Leakiness • Difficult of usage in internal tissues
	CreER^{T2}/LoxP	Lineage tracing Gene overexpression	• Temporal and spatial conditional gene expression • Lineage trace studies	• Permanent recombination • Requires pharmacological treatment (Tamoxifen) • Can be leaky
	Dre/Rox	Lineage tracing Gene overexpression	• Conditional gene expression • Combined with CreER^{T2}/loxP, allows for binary recombination studies • Dre can be linked to an ER to make it inducible	• Permanent recombination • Few lines available
	TetOn/TetOff	Lineage tracing Gene overexpression	• Specific gene visualization and/or overexpression in a temporal- and spatially controlled manner • Can be turned on and off (non-permanent)	• Doxycyclin toxicity not fully characterized
	Heatshock promoter	Lineage tracing Gene overexpression	• Temperature-dependent inducible expression. • Does not require pharmacological induction	• Gene levels are temperature dependent: precise temperature control is necessary for the method to work • Promotor efficiency varies with cell types
	UAS/Gal4	Cell/ tissue imaging Gene overexpression	• Spatially controlled gene expression	• Transcriptional silencing in very active promoters
	TALEN	Genome editing	• Highly specific and efficient • More versatile for sequence recognition than CRISPR	• Time consuming
	CRISPR/Cas9	Genome editing	• sgRNA easier to produce than TALEN • Targeting of various genomic regions with one injection	• Possible off-target effects • Usage for site-directed insertions still not fully established
Delivery methods	Oocyte injection	Delivery of DNA, RNA and proteins	• Flexible and reliable	• Challenging for species with internal fertilization
	Electroporation	Delivery of DNA and RNA	• Flexible	• Electrical charges can damage the tissue • High concentrations of reagents required
	Virus transfection	Delivery of DNA, RNA and proteins	• Efficient, widespread and targeted delivery	• Time consuming • Technically challenging
Imaging techniques	Confocal microscopy	Imaging	• Best for fixed samples	• Sample bleaching • Laser power can damage sample
	LIght-sheet fluorescent microscopy	Live Imaging	• Ideal for live imaging	• Limited to small sized samples
	Two-photon/ multi-photon fluorescent microscopy	Live Imaging	• Ideal for live imaging • Increased tissue penetrance due to increased wavelength used	• Limited to small sized samples
	µCT	Fixed samples Live Imaging	• Live imaging • Ideal for bone image acquisition	• Not indicated for soft tissue

Different mammalian species can regenerate structures composed of different tissue types, which include the digit tip, the ear in rabbits and rodents, and the seasonal regeneration of antlers in deer. The African spiny mouse, a recently established animal model, is contributing critical insights into mammalian

regeneration. In the skin of this small rodent, full-thickness wounds heal leaving no scar, which is thought to be an evolutionary trait to confuse predators (similar to autotomy in lizards). This allows spiny mice to detach pieces of skin while escaping predators in its native habitat (North Africa). Sharing features of other species reviewed in this chapter, injuries in the ear of the African spiny mouse form a blastema that, within weeks, will fill the missing part (Seifert et al. 2012). Furthermore, it has been found that inflammatory cells are essential for ear regeneration (Simkin 2017a).

Finally, aging is a factor associated with a decline of physiological and restorative regeneration. A remarkable display of repair is found in mammalian embryo development, where wounds are healed scarless through mechanisms that are still largely unknown. This trait is common in animal models and humans (Colwell et al. 2005; Rowlatt 1979), and has highlighted the important role of extracellular matrix components and the inflammatory response (reviewed in Larson et al. 2010).

7.1 Digit Tip Regeneration

The fingertip is a unique example of restorative regeneration, and it has been documented in mice, nonhuman primates, and humans. A successful restoration depends primarily on the nail organ and the amputation plane, limiting regeneration to the distal-most part of the third phalanx (Han et al. 2008; Neufeld and Zhao 1995; Reginelli et al. 1995). Mouse digit tip regeneration occurs in phases similar to amphibian limb regeneration: wound closure, blastema formation, and patterning. After injury, once coagulation and vessel constriction stop blood loss, the wound is re-epithelialized. This process, which occurs within hours in amphibians, can take up to 6–8 days in mouse digit tips (Han et al. 2003) and 7–10 days in macaques (Singer et al. 1987). Before the wound is completely closed, macrophages and neutrophils infiltrate the tissue. Later, at 10 days post amputation (dpa), only neutrophils are found throughout the blastema, which return to homeostatic numbers by 21 dpa. Macrophages are essential for the formation of a blastema by secreting cytokines and enzymes that will recruit cells and activate proliferation (Simkin et al. 2017b; Simkin et al. 2017a). During the inflammatory phase, extracellular matrix is degraded by enzymatic activity. This histolytic response is more pronounced in bone, and is driven by a specific cell type that is responsible for its resorption, the osteoclasts (Fernando et al. 2011; Neufeld and Zhao 1995). Reducing bone resorption chemically or with wound occlusion delays regeneration but bone formation is still visible, suggesting bone resorption is not indispensable for a regenerative outcome (Simkin et al. 2015). Whether bone resorption is necessary for releasing cells or factors trapped in the extracellular matrix remains to be elucidated. Increasing evidence from our own lab points at a significant role of this process in the integration of the new tissue with the mature one.

Similar to other vertebrates, the cellular source of the mouse digit blastema resides at the amputation plane and the cells within it have their lineage restricted (Lehoczky et al. 2011; Rinkevich et al. 2014). This means that cells that contribute to

the blastema will remain associated to their tissue of origin, giving rise to progeny that will make up that particular tissue in the regenerate. The implications of these comparisons go beyond being merely complementary studies to amphibians, as they provide important implications for human regeneration.

An important question in the context of digit tip regeneration is: why is the digit tip permissive for regeneration, but amputations at more proximal levels result in scar formation? Cells at the base of the nail have been shown to have an important role in orchestrating regeneration of the mouse digit tip. These self-renewing stem cells that support the continuous growth of the nail are driven by the Wnt family of proteins. Upon amputation, this pathway is triggered and attracts nerves, which in turn secrete Fgf2, activating the underlying mesenchymal cells (Takeo et al. 2013). Both Wnt and Fgf pathways are active in salamander regeneration, highlighting some conservation of regenerative signaling cascades that control cell proliferation. However, transplanting a nail organ to more proximal amputation sites (e.g. P2) only promotes bone growth, and does not form a joint or the third phalanx (Mohammad et al. 1999). While the nail organ has an essential role in digit tip regeneration, additional factors seem to play a role, including the immune system and the mechanisms responsible for the suppression of fibrosis in the digit tip.

As such, the study of digit tip regeneration has provided many clues about mammalian regeneration, and continues to be the paradigm to study how to induce regeneration.

7.2 Human Fingertip Regeneration

The earliest record of human regeneration was reported in 1932 (McKim 1932), in which an adult patient had the distal phalanx removed to avoid the spreading of an infection further in the digit. A more recent study that included patients with ages ranging from 15 to 90 years of age reported successful fingertip regeneration in most patients in up to 12 weeks, which even encompassed the formation of new bone (Lee et al. 1995). Most of the literature is, however, based on pediatric cases, which is likely due to the high abundance of injuries and the shorter time required by children to heal. Among these, the most common injury types reported are crushing and amputation by sharp objects.

Skin flaps and allografts are rather inhibitory of the regenerative process. However, replantation can succeed if the excised fragment is well conserved and the health of the patient does not compromise revascularization (Tang et al. 2014).

Conservative treatment is a permissive treatment that allows regeneration of the fingertips (Douglas 1972; Illingworth 1974). With the conservative treatment, pediatric finger injuries reach epithelialization following 20 to 36 days and show no severe complications or infections (Schultz and Fitze 2018). Although human regeneration has long been reported, the underlying mechanisms have been unreachable to the researcher. Whether there is a recapitulation of the mechanisms observed in animal models, or if human regeneration has evolved independently, is still a matter of debate. Although appendage regeneration in humans appears similar

to rodents, as it is restricted to the last phalanx, the role of the nail organ is not fully understood. In fact, some pediatric cases have shown that even substantial damage to the nail organ still did not prevent the regeneration of the fingertip (Illingworth 1974).

Even though there is a clear and significant regenerative potential in humans, regenerative medicine still faces the future challenge of developing novel therapies that profit from this potential.

8 Conclusions

Our understanding of regeneration has dramatically broadened with the availability of emerging genomic and imaging tools. The Omics age has made possible the large-scale analysis of entire regulatory systems. But, more importantly, the increase in critical mass of researchers in the field, as well as the community resources built around it, was and continues to be crucial for the advancement of regenerative medicine.

The study of animal models has revealed commonalities in organ and organismic regeneration, from cellular lineage restriction to axial patterning. It has, additionally, provided insights into alternatives and adaptive mechanisms that equally result in successful regeneration. Subtle or extreme degrees of regeneration among related species, or position-restricted regeneration (like in the fingertip), can provide the experimental field with what drives or inhibits a structure from restoring itself.

One of the challenges that remains ahead is the amalgamation of animal models and clinical findings to readily address the next steps: How to direct an adult cell to become a blastema-like cell? How to overcome the immune system hurdles that halt regeneration? How to trigger a regenerative response in multiple tissues at the same time? How to increase the tolerance and integration of cellular and biocomposite replacement?

Answering all these questions may only be possible in the very distant future but, during the long process of discovery in this broad scope, efforts can be directed to enhance healing in more modest contexts of injury and disease.

Acknowledgments We thank the members of the Sandoval-Guzmán lab for their support. We thank the CRTD TU Dresden, Alexander von Humboldt Foundation, and DFG for funding the work of our laboratory.

References

Al Haj Baddar NW, Chithrala A, Voss SR (2019) Amputation-induced reactive oxygen species signaling is required for axolotl tail regeneration. Dev Dyn 248:189–196

Albert P, Boilly B, Courty J, Barritault D (1987) Stimulation in cell culture of mesenchymal cells of newt limb blastemas by EDGF I or II (basic or acidic FGF). Cell Differ 21:63–68

Anchelin M, Murcia L, Alcaraz-Perez F, Garcia-Navarro EM, Cayuela ML (2011) Behaviour of telomere and telomerase during aging and regeneration in zebrafish. PLoS One 6(2):e16955

Athippozhy A, Lehrberg J, Monaghan JR, Gardiner DM, Voss SR (2014) Characterization of in vitro transcriptional responses of dorsal root ganglia cultured in the presence and absence of blastema cells from regenerating salamander limbs. Regeneration 1:1–10
Aztekin C, Hiscock TW, Marioni JC, Gurdon JB, Simons BD, Jullien J (2019) Identification of a regeneration-organizing cell in the Xenopus tail. Science 364:653–658
Baguñà J (2012) The planarian neoblast: the rambling history of its origin and some current black boxes. Int J Dev Biol 56:19–37
Bartra R, Viñas GV (2011) Axolotiada. Fondo de Cultura Economica USA
Becker CG, Becker T (2008) Adult zebrafish as a model for successful central nervous system regeneration. Restor Neurol Neurosci 26:71–80
Berberoglu MA, Gallagher TL, Morrow ZT, Talbot JC, Hromowyk KJ, Tenente IM, Langenau DM, Amacher SL (2017) Satellite-like cells contribute to pax7-dependent skeletal muscle repair in adult zebrafish. Dev Biol 424:162–180
Brandon RA (1972) Hybridization between the Mexican Salamanders Ambystoma dumerilii and Ambystoma mexicanum under laboratory conditions. Herpetologica 28:199–207
Braun E, Keren K (2018) HydraRegeneration: closing the loop with mechanical processes in morphogenesis. BioEssays 40:1700204–1700212
Brockes JP (2015) Variation in salamanders: an essay on genomes, development, and evolution. In: Kumar A, Simon A (eds) Salamanders in regeneration research: methods and protocols. Springer New York, New York, pp 3–15
Brunauer R, Muneoka K (2018) The impact of aging on mechanisms of mammalian epimorphic regeneration. Gerontology 64(3):300–308, 1–9
Bryant SV, Gardiner DM (2002) Vertebrate limb regeneration and the origin of limb stem cells. Int J Dev Biol 46(7):887–896, 1–10
Bryant DM, Sousounis K, Payzin-Dogru D, Bryant S, Sandoval AGW, Martinez Fernandez J, Mariano R, Oshiro R, Wong AY, Leigh ND et al (2017a) Identification of regenerative roadblocks via repeat deployment of limb regeneration in axolotls. NPJ Regen Med 2:30
Bryant DM, Johnson K, DiTommaso T, Tickle T, Couger MB, Payzin-Dogru D, Lee TJ, Leigh ND, Kuo TH, Davis FG et al (2017b) A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. Cell Rep 18:762–776
Butler EG (1933) Regeneration of the fore limb. J Exptl Zool 65:271–315
Butler EG, O'Brien JP (1942) Effects of localized x-radiation on regeneration of the urodele limb. Anat Rec 84:407–413
Buzgariu W, Wenger Y, Tcaciuc N, Catunda-Lemos A-P, Galliot B (2018) Impact of cycling cells and cell cycle regulation on Hydra regeneration. Dev Biol 433:240–253
Carlson MRJ, Bryant SV, Gardiner DM (1998) Expression of Msx-2 during development, regeneration, and wound healing in axolotl limbs. J Exp Zool 282:715–723
Carr MJ, Toma JS, Johnston APW, Steadman PE, Yuzwa SA, Mahmud N, Frankland PW, Kaplan DR, Miller FD (2019) Mesenchymal precursor cells in adult nerves contribute to mammalian tissue repair and regeneration. Cell Stem Cell 24:240–256.e9
Carroll RL (2007) The palaeozoic ancestry of salamanders, frogs and caecilians. Zool J Linnean Soc 150:1–140
Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, Rattei T, Balasubramanian PG, Borman J, Busam D et al (2010) The dynamic genome of Hydra. Nature 464:592–596
Chen WCW, Wang Z, Missinato MA, Park DW, Long DW, Liu HJ, Zeng X, Yates NA, Kim K, Wang Y (2016) Decellularized zebrafish cardiac extracellular matrix induces mammalian heart regeneration. Sci Adv 2(11):e1600844
Chew KE, Cameron JA (1983) Increase in mitotic activity of regenerating axolotl limbs by growth factor-impregnated implants. J Exp Zool 226:325–329
Christensen RN, Weinstein M, Tassava RA (2002) Expression of fibroblast growth factors 4, 8, and 10 in limbs, flanks, and blastemas of Ambystoma. Dev Dyn 223:193–203
Collins JP (1981) Distribution, habitats and life history variation in the tiger salamander, Ambystoma tigrinum, in East-Central and Southeast Arizona published by: American Society

of Ichthyologists and Herpetologists Distribution, Habitats and Life History Variation. Copeia 1981:666–675
Colwell AS, Longaker MT, Lorenz HP (2005) Mammalian fetal organ regeneration. Adv Biochem Eng Biotechnol 93:83–100
Contreras V, Mart i nez-Meyer E, Valiente E, Zambrano L (2009) Recent decline and potential distribution in the last remnant area of the microendemic Mexican axolotl (Ambystoma mexicanum). Biol Conserv 142:2881–2885
Crawford K, Stocum DL (1988) Retinoic acid proximalizes level-specific properties responsible for intercalary regeneration in axolotl limbs. Development 104:703–712
Currie JD, Kawaguchi A, Traspas RM, Schuez M, Chara O, Tanaka EM (2016) Live imaging of axolotl digit regeneration reveals spatiotemporal choreography of diverse connective tissue progenitor pools. Dev Cell 39:411–423
Currie JD, Grosser L, Murawala P, Schuez M, Michel M, Tanaka EM, Sandoval-Guzmán T (2019) The Prrx1 limb enhancer marks an adult subpopulation of injury-responsive dermal fibroblasts. Biol Open 8:bio043711
Da Silva SM, Gates PB, Brockes JP (2002) The newt ortholog of CD59 is implicated in proximodistal identity during amphibian limb regeneration. Dev Cell 3:547–555
Denis J-F, Sader F, Gatien S, Villiard É, Philip A, Roy S (2016) Activation of Smad2 but not Smad3 is required to mediate TGF-β signaling during axolotl limb regeneration. Development (Cambridge) 143:3481–3490
Dinsmore CE (2007) A history of regeneration research. Cambridge University Press, Cambridge
Douglas BS (1972) Conservative management of guillotine amputation of finger in children. Aust Paediatr J 8:86–89
Dunis DA, Namenwirth M (1977) The role of grafted skin in the regeneration of X-irradiated axolotl limbs. Dev Biol 56:97–109
Echeverri K, Tanaka EM (2005) Proximodistal patterning during limb regeneration. Dev Biol 279:391–401
Endo T, Bryant SV, Gardiner DM (2004) A stepwise model system for limb regeneration. Dev Biol 270:135–145
Enyedi B, Jelcic M, Niethammer P (2016) The cell nucleus serves as a mechanotransducer of tissue damage-induced inflammation. Cell 165:1160–1170
Farkas JE, Monaghan JR (2017) A brief history of the study of nerve dependent regeneration. Neurogenesis 4:1–12
Farkas JE, Freitas PD, Bryant DM, Whited JL, Monaghan JR (2016) Neuregulin-1 signaling is essential for nerve-dependent axolotl limb regeneration. Development 143:2724–2731
Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39:1477–1487
Fernando WA, Leininger E, Simkin J, Li N, Malcom CA, Sathyamoorthi S, Han M, Muneoka K (2011) Wound healing and Blastema formation in regenerating digit tips of adult mice. Dev Biol 350:301–310
Ferris DR, Satoh A, Mandefro B, Cummings GM, Gardiner DM, Rugg EL (2010) Ex vivo generation of a functional and regenerative wound epithelium from axolotl (Ambystoma mexicanum) skin. Develop Growth Differ 52:715–724
Fröbisch NB, Shubin NH (2011) Salamander limb development: integrating genes, morphology, and fossils. Dev Dyn 240:1087–1099
Fröbisch NB, Bickelmann C, Witzmann F (2014) Early evolution of limb regeneration in tetrapods: evidence from a 300-million-year-old amphibian. Proc R Soc B Biol Sci 281(1794):20141550
Frostick SP, Yin Q, Kemp GJ (1998) Schwann cells, neurotrophic factors, and peripheral nerve regeneration. Microsurgery 18:397–405
Galliot B, Ghila L (2010) Cell plasticity in homeostasis and regeneration. Mol Reprod Dev 77:837–855
Gao K-Q, Shubin NH (2003) Earliest known crown-group salamanders. Nature 422:424–428
Gaviño MA, Reddien PW (2011) A Bmp/Admp regulatory circuit controls maintenance and regeneration of dorsal-ventral polarity in planarians. Curr Biol 21:294–299

Gemberling M, Bailey TJ, Hyde DR, Poss KD (2013) The zebrafish as a model for complex tissue regeneration. Trends Genet 29:611–620
Gerber T, Murawala P, Knapp D, Masselink W, Schuez M, Hermann S, Gac-Santel M, Nowoshilow S, Kageyama J, Khattak S et al (2018) Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. Science 362(80):eaaq0681
Geurtzen K, Knopf F, Wehner D, Huitema LFA, Schulte-Merker S, Weidinger G (2014) Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull. Development 141:2225–2234
Gierer A, Berking S, Bode H, David NC, Flick K, Hansmann G, Schaller H, Trenkner E (1972) Regeneration in hydra from reaggregated cells. Nat New Biol 239:98–101
Gilbert SF (1997) Developmental biology. Sinauer Associates, Sunderland
Globus M, Smith MJ, Vethamany-Globus S (1991) Evidence supporting a mitogenic role for substance P in amphibian limb regeneration. Ann N Y Acad Sci 632:396–399
Godwin JW, Pinto AR, Rosenthal NA (2013) Macrophages are required for adult salamander limb regeneration. Proc Natl Acad Sci U S A 110:9415–9420
Godwin J, Kuraitis D, Rosenthal N (2014) Extracellular matrix considerations for scar-free repair and regeneration: insights from regenerative diversity among vertebrates. Int J Biochem Cell Biol 56:47–55
Goldhamer DJ, Tassava RA (1987) An analysis of proliferative activity in innervated and denervated forelimb regenerates of the newt, Notophthalmus viridescens. Development 100:619–628
Goss RJ (1956a) The regenerative responses of amputated limbs to delayed insertion into the body cavity. Anat Rec 126:283–297
Goss RJ (1956b) Regenerative inhibition following limb amputation and immediate insertion into the body cavity. Anat Rec 126:15–27
Grohme MA, Schloissnig S, Rozanski A, Pippel M, Young GR, Winkler S, Brandl H, Henry I, Dahl A, Powell S et al (2018) The genome of Schmidtea mediterranea and the evolution of core cellular mechanisms. Nature 554:56–61
Han MJ, An JY, Kim W-S (2001) Expression patterns of Fgf-8 during development and limb regeneration of the axolotl. Dev Dyn 220:40–48
Han M, Yang X, Farrington JE, Muneoka K (2003) Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. Development 130:5123–5132
Han M, Yang X, Lee J, Allan CH, Muneoka K (2008) Development and regeneration of the neonatal digit tip in mice. Dev Biol 315:125–135
Hasegawa T, Hall CJ, Crosier PS, Abe G, Kawakami K, Kudo A, Kawakami A (2017) Transient inflammatory response mediated by interleukin-1β is required for proper regeneration in zebrafish fin fold. elife 6:1–22
Hay ED (1958) The fine structure of blastema cells and differentiating cartilage cells in regenerating limbs of Amblystoma larvae. J Biophys Biochem Cytol 4:583–591
Hay ED, Fischman DA (1961) Origin of the Blastema in regenerating limbs of the Newt Triturus viridescens. Dev Biol:1–34
Hervera A, De Virgiliis F, Palmisano I, Zhou L, Tantardini E, Kong G, Hutson T, Danzi MC, Perry RB-T, Santos CXC et al (2018) Reactive oxygen species regulate axonal regeneration through the release of exosomal NADPH oxidase 2 complexes into injured axons. Nat Cell Biol 20:307–319
Iglesias M, Gomez-Skarmeta JL, Saló E, Adell T (2008) Silencing of Smed-betacatenin1 generates radial-like hypercephalized planarians. Development 135:1215–1221
Illingworth CM (1974) Trapped fingers and amputated finger tips in children. J Pediatr Surg 9:853–858
Iten LE, Bryant SV (1975) The interaction between the blastema and stump in the establishment of the anterior-posterior and proximal-distal organization of the limb regenerate. Dev Biol 44:119–147
Ivankovic M, Haneckova R, Thommen A, Grohme MA, Vila-Farré M, Werner S, Rink JC (2019) Model systems for regeneration: planarians. Development 146:dev167684–12
Johnston APW, Yuzwa SA, Carr MJ, Mahmud N, Storer MA, Krause MP, Jones K, Paul S, Kaplan DR, Miller FD (2016) Dedifferentiated schwann cell precursors secreting paracrine factors are required for regeneration of the mammalian digit tip. Cell Stem Cell 19:433–448

Jopling C, Sleep E, Raya M, Martí M, Raya A, Belmonte JCI (2010) Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature:1–6

Kawakami Y, Rodriguez Esteban C, Raya M, Kawakami H, Marti M, Dubova I, Izpisua Belmonte JC (2006) Wnt/beta-catenin signaling regulates vertebrate limb regeneration. Genes Dev 20:3232–3237

Kiffmeyer WR, Tomusk EV, Mescher AL (1991) Axonal transport and release of transferrin in nerves of regenerating amphibian limbs. Dev Biol 147:392–402

Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, MacRae CA, Stainier DYR, Poss KD (2010) Primary contribution to zebrafish heart regeneration by gata4+ cardiomyocytes. Nature 464(7288):601–605,1–7

Knapp D, Tanaka EM (2012) Regeneration and reprogramming. Curr Opin Genet Dev 22:485–493

Knopf F, Hammond C, Chekuru A, Kurth T, Hans S, Weber CW, Mahatma G, Fisher S, Brand M, Schulte-Merker S et al (2011) Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. Dev Cell 20:713–724

Kragl M, Knapp D, Nacu E, Khattak S, Maden M, Epperlein HH, Tanaka EM (2009) Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature 460:60–65

Kroehne V, Freudenreich D, Hans S, Kaslin J, Brand M (2011) Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. Development 138:4831–4841

Kumar A, Godwin JW, Gates PB, Garza-Garcia AA, Brockes JP (2007) Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. Science 318(80):772–777

Larson BJ, Longaker MT, Lorenz HP (2010) Scarless fetal wound healing: a basic science review. Plast Reconstr Surg 126:1172–1180

Lee LP, Lau PY, Chan CW (1995) A simple and efficient treatment for fingertip injuries. J Hand Surg Br 20:63–71

Lehoczky JA, Robert B, Tabin CJ (2011) Mouse digit tip regeneration is mediated by fate-restricted progenitor cells. Proc Natl Acad Sci U S A 108:20609–20614

Leigh ND, Dunlap GS, Johnson K, Mariano R, Oshiro R, Wong AY, Bryant DM, Miller BM, Ratner A, Chen A et al (2018) Transcriptomic landscape of the blastema niche in regenerating adult axolotl limbs at single-cell resolution. Nat Commun 9:1–14

Lévesque M, Gatien S, Finnson K, Desmeules S, Villiard É, Pilote M, Philip A, Roy S (2007) Transforming growth factor: β signaling is essential for limb regeneration in axolotls. PLoS One 2:e1227–e1214

Liversage RA, McLaughlin DS (1983) Effects of delayed amputation on denervated forelimbs of adult newt. *J Embryol Exp Morphol*. 75:1–10

Liversage RA, Stewart WE, McLaughlin DS (1984) In vitro studies of the influence of prolactin on tail regeneration in the adult newt Notophthalmus viridescens. Wilhelm Roux's Arch Dev Biol 193:379–387

Love NR, Chen Y, Ishibashi S, Kritsiligkou P, Lea R, Koh Y, Gallop JL, Dorey K, Amaya E (2013) Amputation-induced reactive oxygen species are required for successful Xenopus tadpole tail regeneration. Nat Cell Biol 15:222–228

Loyd RM, Tassava RA (1980) DNA synthesis and mitosis in adult newt limbs following amputation and insertion into the body cavity. J Exp Zool 214:61–69

Maden M (1978) Neurotrophic control of the cell cycle during amphibian limb regeneration. *J Embryol Exp Morphol* 48:169–175

Maden M (1980) Structure of supernumerary limbs. Nature 286:803–805

Maden M (1983) The effect of vitamin A on the regenerating axolotl limb. J Embryol Exp Morphol 77:273–295

Makanae A, Hirata A, Honjo Y, Mitogawa K, Satoh A (2013) Nerve independent limb induction in axolotls. Dev Biol 381:213–226

Makanae A, Mitogawa K, Satoh A (2014) Co-operative Bmp- and Fgf-signaling inputs convert skin wound healing to limb formation in urodele amphibians. Dev Biol 396:57–66

Maki N, Suetsugu-Maki R, Tarui H, Agata K, Del Rio-Tsonis K, Tsonis PA (2009) Expression of stem cell pluripotency factors during regeneration in newts. Dev Dyn 238:1613–1616

Marques IJ, Lupi E, Mercader N (2019) Model systems for regeneration: zebrafish. Development 146:dev167692–13
Matsuda H, Yokoyama H, Endo T, Tamura K, Ide H (2001) An epidermal signal regulates Lmx-1 expression and dorsal-ventral pattern during Xenopus limb regeneration. Dev Biol 229:351–362
McCauley B, Maxwell D, Collard M (2018) A cross-cultural perspective on upper Palaeolithic hand images with missing phalanges. J Paleo Arch 1:1–20
McKim LH (1932) Regeneration of the distal phalanx. Can Med Assoc J 26(5):549–550. 1–2
Mercader N, Tanaka EM, Torres M (2005) Proximodistal identity during vertebrate limb regeneration is regulated by Meis homeodomain proteins. Development 132:4131–4142
Mescher AL (1976) Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. J Exp Zool 195:117–127
Mescher AL, Gospodarowicz D (1979) Mitogenic effect of a growth factor derived from myelin on denervated regenerates of newt forelimbs. J Exp Zool 207:497–504
Mescher AL, Munaim SI (1986) Changes in the extracellular matrix and glycosaminoglycan synthesis during the initiation of regeneration in adult newt forelimbs. Anat Rec 214:424–431
Mescher AL, Tassava RA (1975) Denervation effects on DNA replication and mitosis during the initiation of limb regeneration in adult newts. Dev Biol 44:187–197
Mescher AL, Connell E, Hsu C, Patel C, Overton B (1997) Transferrin is necessary and sufficient for the neural effect on growth in amphibian limb regeneration blastemas. Develop Growth Differ 39:677–684
Miller BM, Johnson K, Whited JL (2019) Common themes in tetrapod appendage regeneration: a cellular perspective. EvoDevo 10:1–13
Miyajima A, Tanaka M, Itoh T (2014) Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming. Cell Stem Cell 14:561–574
Miyaoka Y, Ebato K, Kato H, Arakawa S, Shimizu S, Miyajima A (2012) Hypertrophy and unconventional cell division of hepatocytes underlie liver regeneration. Curr Biol 22:1166–1175
Mohammad KS, Day FA, Neufeld DA (1999) Bone growth is induced by nail transplantation in amputated proximal phalanges. Calcif Tissue Int 65:1–3
Monaghan JR, Maden M (2012) Visualization of retinoic acid signaling in transgenic axolotls during limb development and regeneration. Dev Biol 368:63–75
Monaghan JR, Epp LG, Putta S, Page RB, Walker JA, Beachy CK, Zhu W, Pao GM, Verma IM, Hunter T et al (2009) Microarray and cDNA sequence analysis of transcription during nerve-dependent limb regeneration. BMC Biol 7:1–19
Morgan TH (1901) Regeneration and liability to injury. Science 14:235–248
Mullen LM, Bryant SV, Torok MA, Blumberg B, Gardiner DM (1996) Nerve dependency of regeneration: the role of distal-less and FGF signaling in amphibian limb regeneration. Development 122:3487–3497
Muneoka K, Sassoon D (1992) Molecular aspects of regeneration in developing vertebrate limbs. Dev Biol 152:37–49
Muneoka K, Fox WF, Bryant SV (1986) Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls. Dev Biol 116:256–260
Nachtomy O, Smith JEH (2014) The life sciences in early modern philosophy. Oxford University Press, Oxford
Nacu E, Tanaka EM (2011) Limb regeneration: a new development? Annu Rev Cell Dev Biol 27:409–440
Nacu E, Glausch M, Le HQ, Damanik FFR, Schuez M, Knapp D, Khattak S, Richter T, Tanaka EM (2013) Connective tissue cells, but not muscle cells, are involved in establishing the proximo-distal outcome of limb regeneration in the axolotl. Dev 140:513–518
Nacu E, Gromberg E, Oliveira CR, Drechsel D, Tanaka EM (2016) FGF8 and SHH substitute for anterior-posterior tissue interactions to induce limb regeneration. Nature 533:407–410
Neufeld DA, Aulthouse AL (1986) Association of mesenchyme with attenuated basement membranes during morphogenetic stages of newt limb regeneration. Am J Anat 176:411–421

Neufeld DA, Zhao W (1995) Bone regrowth after digit tip amputation in mice is equivalent in adults and neonates. Wound Repair Regen 3:461–466
Newmark PA, Sánchez Alvarado A (2000) Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. Dev Biol 220:142–153
Newmark PA, Sánchez Alvarado A (2002) Not your father's planarian: a classic model enters the era of functional genomics. Nat Rev Genet 3:210–219
Niethammer P, Grabher C, Look AT, Mitchison TJ (2009) A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature 459:996–999
Nowoshilow S, Schloissnig S, Fei JF, Dahl A, Pang AWC, Pippel M, Winkler S, Hastie AR, Young G, Roscito JG et al (2018) The axolotl genome and the evolution of key tissue formation regulators. Nature 554:50–55
Nye HLD, Cameron JA, Chernoff EAG, Stocum DL (2003) Extending the table of stages of normal development of the axolotl: limb development. Dev Dyn 226:555–560
Odland G, Ross R (1968) Human wound repair. I. Epidermal regeneration. J Cell Biol 39:135–151
O'Neill EM, Schwartz R, Bullock CT, Williams JS, Shaffer HB, Aguilar-Miguel X, Parra-Olea G, Weisrock DW (2013) Parallel tagged amplicon sequencing reveals major lineages and phylogenetic structure in the North American tiger salamander (Ambystoma tigrinum) species complex. Mol Ecol 22:111–129
Osorno R, Tsakiridis A, Wong F, Cambray N, Economou C, Wilkie R, Blin G, Scotting PJ, Chambers I, Wilson V (2012) The developmental dismantling of pluripotency is reversed by ectopic Oct4 expression. TL - 139. Development 139:2288–2298
Park JE, Barbul A (2004) Understanding the role of immune regulation in wound healing. Am J Surg 187:S11–S16
Park HD, Ortmeyer AB, Blankenbaker DP (1970) Cell division during regeneration in Hydra. Nature 227:617–619
Payzin-Dogru D, Whited JL (2018) An integrative framework for salamander and mouse limb regeneration. Int J Dev Biol 62:393–402
Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA (2011) Transient regenerative potential of the neonatal mouse heart. Science 331:1078–1080
Poss KD, Wilson LG, Keating MT (2002) Heart regeneration in zebrafish. Science 298:2185–2188
Poulin ML, Chiu I-M (1995) Re-programming of expression of the KGFR and bek variants of fibroblast growth factor receptor 2 during limb regeneration in newts (Notophthalmus viridescens). Dev Dyn 202:378–387
Poulin ML, Patrie KM, Botelho MJ, Tassava RA, Chiu IM (1993) Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (Notophthalmus viridescens). Development 119:353–361
Pownall ME, Isaacs HV (2010) Limb development. FGF Signalling in Vertebrate Development, Limb Development. Morgan & Claypool Life Sciences. 16:110–123
Rabiela TR (1998) La cosecha del agua en la cuenca de México. CIESAS, México
Rao N, Jhamb D, Milner DJ, Li B, Song F, Wang M, Voss SR, Palakal M, King MW, Saranjami B et al (2009) Proteomic analysis of blastema formation in regenerating axolotl limbs. BMC Biol 7:1–25
Razzell W, Evans IR, Martin P, Wood W (2013) Calcium flashes orchestrate the wound inflammatory response through Duox activation and hydrogen peroxide release. Curr Biol 23:424–429
Reddien PW (2013) Specialized progenitors and regeneration. Development 140:951–957
Reddien PW (2018) The cellular and molecular basis for planarian regeneration. Cell 175:1–19
Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sánchez Alvarado A (2005) Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev Cell 8:635–649
Reginelli AD, Wang YQ, Sassoon D, Muneoka K (1995) Digit tip regeneration correlates with regions of Msx1 (Hox 7) expression in fetal and newborn mice. Development 121:1065–1076
Repesh LA, Oberpriller JC (1980) Ultrastructural studies on migrating epidermal cells during the wound healing stage of regeneration in the adult newt, Notophthalmus viridescens. Am J Anat 159:187–208
Richmond MJ, Pollack ED (1983) Regulation of tadpole spinal nerve fiber growth by the regenerating limb blastema in tissue culture. J Exp Zool 225:233–242

Rink JC (2013) Stem cell systems and regeneration in planaria. Dev Genes Evol 223:67–84
Rinkevich Y, Lindau P, Ueno H, Longaker MT, Weissman IL (2012) Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. Nature 476:409–413
Rinkevich Y, Montoro DT, Muhonen E, Walmsley GG, Lo D, Hasegawa M, Januszyk M, Connolly AJ, Weissman IL, Longaker MT (2014) Clonal analysis reveals nerve-dependent and independent roles on mammalian hind limb tissue maintenance and regeneration. Proc Natl Acad Sci U S A
Roensch K, Tazaki A, Chara O, Tanaka EM (2013) Progressive specification rather than intercalation of segments during limb regeneration. Science 342(80):1375–1379
Roskams TA, Libbrecht L, Desmet VJ (2003) Progenitor cells in diseased human liver. Semin Liver Dis 23:385–396
Rowlatt U (1979) Intrauterine wound healing in a 20 week human fetus. Virchows Arch A Pathol Anat Histol 381:353–361
Roy S, Gardiner DM (2002) Cyclopamine induces digit loss in regenerating axolotl limbs. J Exp Zool 293:186–190
Roy S, Gardiner DM, Bryant SV (2000) Vaccinia as a tool for functional analysis in regenerating limbs: ectopic expression of Shh. Dev Biol 218:199–205
Sader F, Denis J-FXO, Laref H, Roy SXP (2019) Epithelial to mesenchymal transition is mediated by both TGF-β canonical and non-canonical signaling during axolotl limb regeneration. Nat Publ Group 9:1–13
Salpeter MM, Singer M (1960) The fine structure of mesenchymatous cells in the regenerating forelimb of the adult newt Triturus. Dev Biol 2:516–534
Sanchez AA (2006) Planarian regeneration: its end is its beginning. Cell 124:241–245
Sandoval-Guzmán T, Currie JD (2018) The journey of cells through regeneration. Curr Opin Cell Biol 55:36–41
Sandoval-Guzmán T, Wang H, Khattak S, Schuez M, Roensch K, Nacu E, Tazaki A, Joven A, Tanaka EM, Simon A (2014) Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. Cell Stem Cell 14:174–187
Satoh A, Gardiner DM, Bryant SV, Endo T (2007) Nerve-induced ectopic limb blastemas in the axolotl are equivalent to amputation-induced blastemas. Dev Biol 312:231–244
Satoh A, Graham GMC, Bryant SV, Gardiner DM (2008) Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (Ambystoma mexicanum). Dev Biol 319:321–335
Satoh A, Makanae A, Hirata A, Satou Y (2011) Blastema induction in aneurogenic state and Prrx-1 regulation by MMPs and FGFs in Ambystoma mexicanum limb regeneration. Dev Biol 355:263–274
Satoh A, Bryant SV, Gardiner DM (2012) Nerve signaling regulates basal keratinocyte proliferation in the blastema apical epithelial cap in the axolotl (Ambystoma mexicanum). Dev Biol 366:374–381
Satoh A, Mitogawa K, Makanae A (2015) Regeneration inducers in limb regeneration. Develop Growth Differ 57:421–429
Satoh A, Makanae A, Nishimoto Y, Mitogawa K (2016) FGF and BMP derived from dorsal root ganglia regulate blastema induction in limb regeneration in Ambystoma mexicanum. Dev Biol 417:114–125
Scadding SR, Maden M (1994) Retinoic acid gradients during limb regeneration. Dev Biol 162:608–617
Schotté OE, Butler EG (1941) Morphological effects of denervation and amputation of limbs in urodele larvae. J Exp Zool 87:279–322
Schotté OE, Butler EG (1944) Phases in regeneration of the urodele limb and their dependence upon the nervous system. J Exp Zool 97:95–121
Schultz J, Fitze G (2018) Conservative treatment of fingertip injuries in children – first experiences with a novel silicone finger cap that enables woundfluid analysis. 1–12. GMS Interdiscip Plast Reconstr Surg DGPW. 7, Doc5.
Sehring IM, Jahn C, Weidinger G (2016) ScienceDirect zebrafish fin and heart: what's special about regeneration? Curr Opin Genet Dev 40:48–56

Seifert AW, Kiama SG, Seifert MG, Goheen JR, Palmer TM, Maden M (2012) Skin shedding and tissue regeneration in African spiny mice (Acomys). Nature 489:561–565

Shaffer HB (1984) Evolution in a paedomorphic lineage. I. an electrophoretic analysis of the mexican ambystomatid salamanders. Evolution 38:1194–1206

Shaffer HB (1993) Phylogenetics of model organisms: the laboratory axolotl, ambystoma mexicanum. Syst Biol 42:508–522

Shaffer HB, Randal Voss S (1996) Phylogenetic and mechanistic analysis of a developmentally integrated character complex: alternate life history modes in ambystomatid salamanders. Am Zool 36:24–35

Shen XX, Liang D, Feng YJ, Chen MY, Zhang P (2013) A versatile and highly efficient toolkit including 102 nuclear markers for vertebrate phylogenomics, tested by resolving the higher level relationships of the caudata. Mol Biol Evol 30:2235–2248

Simkin J, Seifert AW (2017) Concise review: translating regenerative biology into clinically relevant therapies: are we on the right path? Stem Cells Transl Med 7:220–231

Simkin J, Sammarco MC, Dawson LA, Tucker C, Taylor LJ, Van Meter K, Muneoka K (2015) Epidermal closure regulates histolysis during mammalian (Mus) digit regeneration. Regeneration 2:106–119

Simkin J, Gawriluk TR, Gensel JC, Seifert AW (2017a) Macrophages are necessary for epimorphic regeneration in African spiny mice. elife 6:1–26

Simkin J, Sammarco MC, Marrero L, Dawson LA, Yan M, Tucker C, Cammack A, Muneoka K (2017b) Macrophages are required to coordinate mouse digit tip regeneration. Development 144(21):3907–3916. dev.150086

Singer M (1946) The nervous system and regeneration of the forelimb of adult Triturus. V. The influence of number of nerve fibers, including a quantitative study of limb innervation. J Exp Zool 101:299–337

Singer M (1949) The invasion of the epidermis of the regenerating forelimb of the urodele, Triturus, by nerve fibers. J Exp Zool 111:189–209

Singer M, Craven L (1948) The growth and morphogenesis of the regenerating forelimb of adult Triturus following denervation at various stages of development. J Exp Zool 108:279–308

Singer M, Inoue S (1964) The nerve and the epidermal apical cap in regeneration of the forelimb of adult Triturus. J Exp Zool 155:105–115

Singer M, Weckesser EC, Géraudie J, Maier CE, Singer J (1987) Open finger tip healing and replacement after distal amputation in Rhesus monkey with comparison to limb regeneration in lower vertebrates. Anat Embryol:1–8

Singh SP, Holdway JE, Poss KD (2012) Regeneration of amputated zebrafish fin rays from De Novo Osteoblasts. Dev Cell 22:879–886

Smith AR, Wolpert L (1975) Nerves and angiogenesis in amphibian limb regeneration. Nature 257:224–225

Steen TP, Thornton CS (1963) Tissue interaction in amputated aneurogenic limbs of Ambystoma larvae. J Exp Zool 154:207–221

Stevenson TJ, Vinarsky V, Atkinson DL, Keating MT, Odelberg SJ (2006) Tissue inhibitor of metalloproteinase 1 regulates matrix metalloproteinase activity during newt limb regeneration. Dev Dyn 235:606–616

Stewart S, Stankunas K (2012) Limited dedifferentiation provides replacement tissue during zebrafish fin regeneration. Dev Biol 365:339–349

Stocum DL (1982) Determination of axial polarity in the urodele limb regeneration blastema. J Embryol Exp Morphol 71:193–214

Stocum DL (1984) The urodele limb regeneration blastema. Differentiation 27:13–28

Stocum DL (2017) Mechanisms of urodele limb regeneration. Regeneration 4:159–200

Stückemann T, Cleland JP, Werner S, Vu HT-K, Bayersdorf R, Liu S-Y, Friedrich B, Jülicher F, Rink JC (2017) Antagonistic self-organizing patterning systems control maintenance and regeneration of the anteroposterior axis in planarians. Dev Cell 40:248–263.e4

Sugiura T, Wang H, Barsacchi R, Simon A, Tanaka EM (2016) MARCKS-like protein is an initiating molecule in axolotl appendage regeneration. Nature 531:237–240

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takashima S, Gold D, Hartenstein V (2013) Stem cells and lineages of the intestine: a developmental and evolutionary perspective. Dev Genes Evol 223:85–102
Takeo M, Chou WC, Sun Q, Lee W, Rabbani P, Loomis C, Taketo MM, Ito M (2013) Wnt activation in nail epithelium couples nail growth to digit regeneration. Nature 499:228–232
Tanaka EM (2016) The molecular and cellular choreography of appendage regeneration. Cell 165:1598–1608
Tanaka EM, Drechsel DN, Brockes JP (1999) Thrombin regulates S-phase re-entry by cultured newt myotubes. Curr Biol 9:792–799
Tang JB, Elliot D, Adani R, Saint-Cyr M, Stang F (2014) Repair and reconstruction of thumb and finger tip injuries: a global view. Clin Plast Surg 41:325–359
Tank PW, Carlson BM, Connelly TG (1976) A staging system for forelimb regeneration in the axolotl, Ambystoma mexicanum. J Morphol 150:117–128
Tetteh PW, Basak O, Farin HF, Wiebrands K, Kretzschmar K, Begthel H, van den Born M, Korving J, de Sauvage F, van Es JH et al (2016) Replacement of lost Lgr5-positive stem cells through plasticity of their enterocyte-lineage daughters. Stem Cell 18:203–213
Thornton CS (1957) The effect of apical cap removal on limb regeneration on Ambystoma larvae. J Exp Zool A Ecol Genet Physiolss 134:357–381
Thornton CS (1960) Influence of an eccentric epidermal cap on limb regeneration in Amblystoma larvae. Dev Biol 2:551–569
Thornton CS, Thornton MT (1970) Recuperation of regeneration in denervated limbs of Ambystoma larvae. J Exp Zool 173:293–301
Tihen JA (1958) Comments on the osteology and phylogeny of Ambystomatid salamanders. Bull Florida State Museum Biol Sci 3:1–51
Tornini VA, Puliafito A, Slota LA, Thompson JD, Nachtrab G, Kaushik A-L, Kapsimali M, Primo L, Di Talia S, Poss KD (2016) Live monitoring of blastemal cell contributions during appendage regeneration. Curr Biol 26:2981–2991
Tsai SL, Baselga-Garriga C, Melton DA (2019) Blastemal progenitors modulate immune signaling during early limb regeneration. Development (Cambridge) 146:dev169128
van Es JH, Sato T, van de Wetering M, Lyubimova A, Nee ANY, Gregorieff A, Sasaki N, Zeinstra L, van den Born M, Korving J et al (2012) Dll1(+) secretory progenitor cells revert to stem cells upon crypt damage. Nat Cell Biol 14:1099–1104
Vinarsky V, Atkinson DL, Stevenson TJ, Keating MT, Odelberg SJ (2005) Normal newt limb regeneration requires matrix metalloproteinase function. Dev Biol 279:86–98
Voss SR, Woodcock MR, Zambrano L (2015) A Tale of Two Axolotls. BioScience 65(12):1134–1140
Wade MJ, McKnight ML, Shaffer HB (1994) The effects of kin-structured colonization on nuclear and cytoplasmic genetic diversity. Evolution 48:1114
Wagner DE, Wang IE, Reddien PW (2011) Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science 332:811–816
Wallace H (1972) The components of regrowing nerves which support the regeneration of irradiated salamander limbs. J Embryol Exp Morphol 28:419–435
Wallace HH (1981) Vertebrate limb regeneration. Wiley, Chichester
Weis JS, Weis P (1970) The effect of nerve growth factor on limb regeneration in Ambystoma. J Exp Zool 174:73–78
Weisrock DW, Shaffer HB, Storz BL, Storz SR, Voss SR (2006) Multiple nuclear gene sequences identify phylogenetic species boundaries in the rapidly radiating clade of Mexican ambystomatid salamanders. Mol Ecol 15:2489–2503
Wells JM, Watt FM (2018) Diverse mechanisms for endogenous regeneration and repair in mammalian organs. Nature 557:322–328
Williams JS, Niedzwiecki JH, Weisrock DW (2013) Species tree reconstruction of a poorly resolved clade of salamanders (Ambystomatidae) using multiple nuclear loci. Mol Phylogenet Evol 68:671–682

Wolpert L, Beddington R, Jessell T, Lawrence P, Meyerowitz E, Smith J (2002) Principles of development, 2nd edn. Oxford University Press, Oxford

Yahalom-Ronen Y, Rajchman D, Sarig R, Geiger B, Tzahor E (2015) Reduced matrix rigidity promotes neonatal cardiomyocyte dedifferentiation, proliferation and clonal expansion. elife 4:1–18

Yang EV, Byant SV (1994) Developmental regulation of a matrix metalloproteinase during regeneration of axolotl appendages. Dev Biol 166:696–703

Yang EV, Gardiner DM, Carlson MR, Nugas CA, Bryant SV (1999) Expression of Mmp-9 and related matrix metalloproteinase genes during axolotl limb regeneration. Dev Dyn 216:2–9

Yntema CL (1959) Regeneration in sparsely innervated and aneurogenic forelimbs of Amblystoma larvae. J Exp Zool 140:101–123

Yokoyama H, Ogino H, Stoick-Cooper CL, Grainger RM, Moon RT (2007) Wnt/β-catenin signaling has an essential role in the initiation of limb regeneration. Dev Biol 306:170–178

Yu L, Han M, Yan M, Lee E-C, Lee J, Muneoka K (2010) BMP signaling induces digit regeneration in neonatal mice. Development 137:551–559

Yun M (2015) Changes in regenerative capacity through lifespan. IJMS 16:25392–25432

Zhu W, Pao GM, Satoh A, Cummings G, Monaghan JR, Harkins TT, Bryant SV, Randal Voss S, Gardiner DM, Hunter T (2012) Activation of germline-specific genes is required for limb regeneration in the Mexican axolotl. Dev Biol 370:42–51

Singer M, Weckesser EC, Géraudie J, Maier CE, Singer J (1987) Open finger tip healing and 1660 replacement after distal amputation in Rhesus monkey with comparison to limb regeneration in 1661 lower vertebrates. Anat Embryol:1–8

Biological Augmentation for Tendon Repair: Lessons to Be Learned from Development, Disease, and Tendon Stem Cell Research

Herbert Tempfer, Christine Lehner, Moritz Grütz, Renate Gehwolf, and Andreas Traweger

Contents

H. Tempfer (✉) · A. Traweger
Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria

University of Salzburg, Salzburg, Austria
e-mail: herbert.tempfer@pmu.ac.at; andreas.traweger@pmu.ac.at

C. Lehner · M. Grütz · R. Gehwolf
Institute of Tendon and Bone Regeneration, Paracelsus Medical University, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Austria, Salzburg, Austria

Austrian Cluster for Tissue Regeneration, Vienna, Austria
e-mail: christine.lehner@pmu.ac.at; moritz.gruetz@pmu.ac.at; renate.gehwolf@pmu.ac.at

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_54

Abstract

Tendons resemble connective tissues rich in highly organized collagen fibers, displaying a remarkably high tensile strength. However, partly due to the low number of tissue-resident cells and their more or less avascular nature, tendons heal relatively slowly. As there is a growing socio-economic need for effective and reproducible treatments to repair injured tendons, researchers and clinicians are challenged to develop strategies to restore native tendon structure and functionality.

This chapter highlights the features and functions of tendon-resident cells and their niche, beginning with a general view on tendon structure. It further gives an overview of tendon development and the cellular and molecular events underlying tendon aging. Finally, we will close the chapter by briefly outlining current strategies to augment tendon repair, aiming at reaching the ambitious goal of functional tendon regeneration.

1 Introduction

Tendons enable musculoskeletal forces to be transmitted and redirected across skeletal joints, facilitating a wide range of joint motion and locomotor movement. Due to their remarkable tensile strength, stiffness, and viscoelastic properties, tendons not only allow the safe transmission of muscle forces over long lengths, but partially also enable the storage and release of elastic energy, reducing energy costs and minimizing the risk of injury.

Tendon disorders are frequent, debilitating conditions affecting both the working population and recreational athletes, placing an enormous burden on healthcare systems worldwide. Despite the high prevalence of tendon injuries due to overuse and/or aging (Maffulli et al. 2003), possible therapeutic interventions remain limited compared to other musculoskeletal tissues, such as bone, muscle, or cartilage. Acute tendon injuries and chronic tendinopathies remain clinically challenging, in part due to very few of low activity tendon-resident cells. Further, the avascular nature of tendons delays healing, while the innate reparative processes are incomplete and often are associated with the formation of scar tissue that compromises the mechanical function (Benjamin and Ralphs 1997). Despite significant advancements in tissue engineering (e.g., sophisticated combination of scaffolds, cells biologics), the clinical impact for the functional regeneration of tendons remains limited. Currently, tendon injuries are either treated by a conservative approach (e.g., eccentric training, pain management) or by surgical intervention. However, irrespective of the methods employed, tendons heal slowly and rarely full function is regained due to the

formation of scar tissue, the formation of adhesions, or ectopic bone formation. To that end, for the development of functional reparative tendon therapies, we need to pin down the molecular and cellular mechanisms amenable to modulate endogenous (or exogenous) cell behavior towards functional tendon repair and regeneration.

Advancements in the fields of biomaterial and stem cell research present promising avenues for the development of alternative treatments. While autografts remain the gold standard for tendon repair augmentation, allografts, xenografts, and synthetic materials are gaining more interest to overcome limited supply and donor site morbidity associated with autograft harvesting (Lomas et al. 2015). However, despite promising preclinical results, scaffold materials do not fully recapitulate native tendon structure, biomechanical properties, or overall composition. Alternatively, the use of adult stem cell therapies has received tremendous attention hoping to successfully repair and/or replace injured or damaged tendon tissue (Lui 2015; Veronesi et al. 2016), as the investigation of the heterogeneous population of tendon-resident cells continues to provide valuable insights into the cellular and molecular mechanism driving tendon disease and healing. The bulk of cells present in the tendon proper are elongated, specialized fibroblast-like cells, termed tenocytes, and their precursor cells, termed tenoblasts (Kannus 2000). In addition, tendons harbor a population of tendon stem and progenitor cells (TSPCs) (Bi et al. 2007; Salingcarnboriboon et al. 2003; Tempfer et al. 2009), synovial cells located in the connective tissue sheaths surrounding the tendon (Banes et al. 1988), and vascular endothelial cells (Lehner et al. 2016). The multipotency and high proliferation rate of TSPCs makes them attractive candidates for tendon repair; however, a better understanding of their functions in situ in health and disease is needed to fully harness their therapeutic potential.

Finally, by investigating the developmental programs driving tendon tissue formation and, on the other hand, the mechanisms contributing to the senescence of tendons, ultimately resulting in decreased quality of tendons in the elderly, novel targets for clinical intervention potentially can be discovered.

2 Tendon Structure and ECM

Tendons contain a range of fibrous, soft tissue structures, endowing them with their biomechanical properties to transmit force from muscle to bone. Their ability to provide rigidity combined with flexibility is made possible by the nonlinear, viscoelastic properties, the biomechanical behavior reflecting the properties of their main building block – type I collagen fibrils. Tendons are organized in a highly hierarchical manner with collagen being bundled into progressively larger subunits (see Fig. 1). The smallest units are the collagen molecules – after secretion from tendon-resident cells, the procollagen molecules are being processed and 300-nm-long triple-helical molecules remain. These molecules then undergo a self-assembly process leading to a staggered arrangement of parallel molecules, with a periodicity of $D = 67$ nm, also known as the "D" period (Canty and Kadler 2002). These insoluble collagen molecules then assemble into microfibrils, which in turn form larger, moderately twisted, lattice-type fibrils by lateral and longitudinal stacking ranging in size from

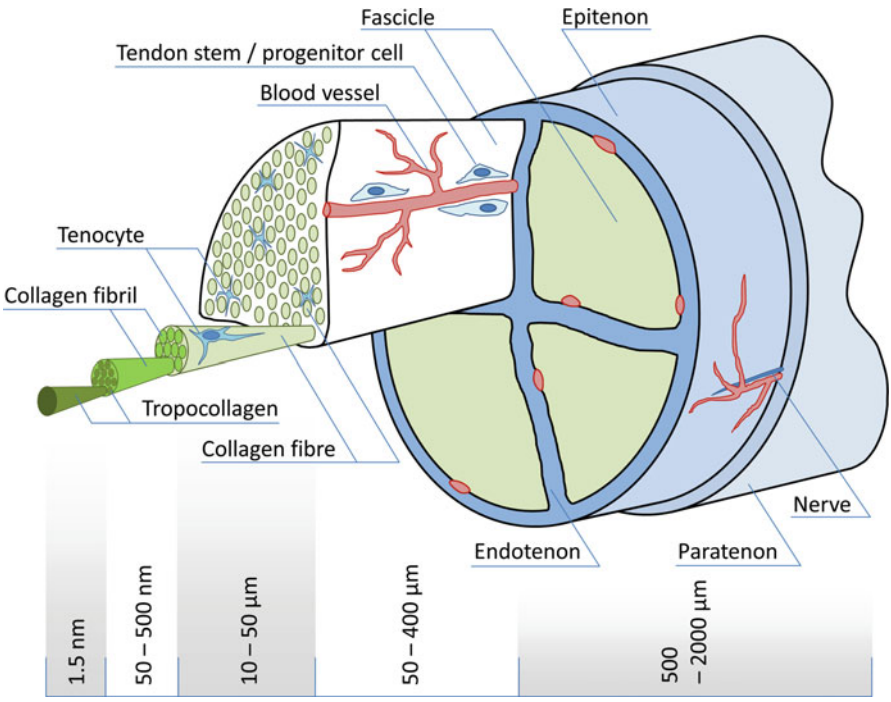


Fig. 1 Hierarchical tendon structure in which mainly collagen type I molecules assemble to form increasingly large subunits of the anisotropic tendon ECM. Tendons are further mainly populated by terminally differentiated tenocytes and a small population of tendon stem and progenitor cells. The tendon fiber bundles are further ensheathed in a loose connective tissue termed endotenon, harboring vessels, lymphatics, and nerves. Finally, the entirety of the tendon is surrounded by the epitenon and paratenon

approximately 10 nm to 500 nm (Canty and Kadler 2005). Fibrils continue to pack together to larger fibers, which are then bound together to fascicles. At each level of this hierarchy, collagens are interspersed with a varying amount of matrix rich in proteoglycans which also significantly contribute to the mechanical behavior of tendons (Thorpe et al. 2013). Finally, fascicles are then bundled by a connective tissue sheath termed the endotenon, forming the intrasubstance, and the epitenon, which encircles the periphery of the full tendon. Both facilitate and lubricate tendon movement and embed blood vessels, nerves, and lymphatics which run to the deeper portion of the tendon (Benjamin et al. 2008).

The majority of the tendon matrix is comprised by collagens, elastin, proteoglycans (PGs), and glycosaminoglycans (GAGs). Collagen type I is the major constituent, accounting for around 60% of the dry mass and about 95% of the total collagen (Sharma and Maffulli 2006; Wang 2006). Other less abundant collagen isotypes include collagen types III, V, VI, XI, XII, and XIV. In healthy tendons, collagen type III is mainly found in the endotenon and epitenon (Kannus 2000). It is thought to be essential for collagen fibril formation regulating collagen type I fibril size (Kadler

et al. 1990). Further, an increase of collagen type III has been reported for overloaded or injured tendons (Pajala et al. 2009; Pingel et al. 2014), and it is believed to have a role in the healing response. Collagen type V and the nonfibrillar collagens XII and XIV also serve a regulatory role during fibrillogenesis (Ansorge et al. 2009; Wenstrup et al. 2004). Elastin is present in tendon as elastic fibers which make up around 1–2% of the total dry mass (Kannus 2000), providing tissue flexibility, extensibility, and passive recoil (Kielty et al. 2002).

Next to collagens, tenocytes also produce glycoproteins and proteoglycans (PG). PGs are core proteins attached to one or several polysaccharide chains, commonly referred to glycosaminoglycans (GAGs). Generally, in the tensile region of tendons, the majority of PGs belong to the family of small leucine-rich proteoglycans (SLRPs), including decorin, biglycan, fibromodulin, and lumican. SLRPs bind to collagen molecules of fibrils and facilitate fibril assembly (Thorpe et al. 2013). For example, mice lacking decorin, which is the most abundant PG in tendons, develop structurally impaired tendons with irregular fibrils. A rather similar phenotype is seen for biglycan knockout mice (Gordon et al. 2015; Robinson et al. 2005). Interestingly, PG concentrations and type differ for tendons loaded in tension, compression, and shear (Berenson et al. 1996; Riley et al. 1994), and also quantitative difference has been demonstrated within a single tendon, comparing the compressed regions and the tensile regions (Carvalho et al. 2000; Vogel et al. 1993). Fibromodulin (Fmod) and lumican are also involved in collagen fibrillogenesis, potentially stabilizing small-diameter fibrils by preventing their fusion (Chakravarti 2002). Fmod and biglycan have further been shown to be important for the formation and maintenance of the TSPC niche (Bi et al. 2007).

Besides SLRPs, other glycoproteins are important constituents of tendons, such as cartilage oligomeric matrix protein (COMP), fibronectin, tenascin C (TNC), and tenomodulin (Tnmd). Although COMP, a large pentameric protein providing a link to neighboring collagen fibrils (Smith et al. 1997), is the most abundant glycoprotein in tendons, its function remains largely unclear. Tnmd is a type II transmembrane glycoprotein predominantly expressed in tendons and ligaments, but can also be found in other tissue types such as muscle, skin, fat, or nervous tissue (Dex et al. 2016). Next to Scleraxis (Scx) and mohawk homeobox (Mkx), tenomodulin has received increasing attention as a putative tendon-marker protein. Further, Tnmd knockout mice show a reduced proliferation rate and an abnormal collagen fibril phenotype with pathologically thicker fibrils (Docheva et al. 2005).

The physicochemical properties of the SLRPs and PGs allow water uptake, which makes up to 60–80% of the total weight of tendons, whereas proteoglycans themselves account only for 1–2% (Kannus 2000). Generally, tendons grow stiffer as they mature due to increased collagen fibril thickness and the formation of covalent cross-links, which are primarily driven by the enzyme lysyl oxidase (Lox) (Bailey 2001). Beyond these enzymatic cross-links, SLRPs have been demonstrated to directly bond collagen fibrils together or to influence Lox-driven cross-linking reactions (Kalamajski et al. 2014).

Taken together, the structure and composition of a tendon is tightly linked to its function and the hierarchical structure exhibiting a highly anisotropic, nonlinear, and viscoelastic mechanical behavior is fundamental for normal tendon function.

3 Tendon-Resident Cells

Mature tendons are populated by several cell types and so far, mainly due to the lack of reliable tendon-specific markers, our knowledge about their identity remains incomplete. The primary resident cells are tendon fibroblasts, termed tenocytes, which comprise approximately 90% of the tendon cellular compartment (Kannus 2000). These flat, elongated differentiated cells are proposed to arise from tenoblasts (Chuen et al. 2004). However, tenoblasts have also been regarded as an activated form of tenocytes in the case of intrinsic healing of tendon injuries (Davidson et al. 1997). The remaining 10% is composed of synovial cells from the endo−/epitenon, chondrocytes located in close proximity of tendon-to-bone insertions (entheses), and vascular endothelial cells. In addition, a population of multipotent tendon stem and progenitor cells (TSPCs) has been identified in tendons (Bi et al. 2007; Salingcarnboriboon et al. 2003; Tempfer et al. 2009).

TSPCs have been described to reside in two different locations. Most studies describe a population of adult stem cells within the tendon proper, suggesting a mainly nonvascular source of stem cells in tendons (Bi et al. 2007; Lui 2013). In a subsequent study, Mienaltowski et al. reported the isolation of stem and progenitor cells from the peritenon of mouse Achilles tendons (Mienaltowski et al. 2013). Most of these data are deduced from in vitro experiments by isolating TSPCs from different tendon regions and subsequent analysis of clonogenicity, self-renewal capacity, multilineage differentiation potential, and BrdU or IdU-retention over time indicating slow cycling cells which is an accepted stem cell feature (Bi et al. 2007). Generally, adult stem cells often reside in a perivascular niche, and indeed in human supraspinatus tendons a population of perivascular cells expressing tendon and stem cell markers has been described (Tempfer et al. 2009). However, studies unequivocally demonstrating the exact location of TSPCs during tendon maintenance and healing are urgently required (see also further below – tendon stem cell niche).

TSPCs exhibit classical adult mesenchymal stromal cell (MSC) criteria and have been described for human, equine, bovine, rabbit, rat, and mouse tendons. They express specific surface antigens and show self-renewal, clonogenicity, and trilineage differentiation capacity, giving rise to adipogenic, osteogenic, and chondrogenic cells. They meet the marker panel defined by the International Society for Cellular Therapy for MSCs as they express CD90, CD73, and CD105 but are negative for CD31, CD34, CD45, HLA-DR, CD11b, CD14, and CD19. In addition, they express tendon-related proteins such as scleraxis and tenomodulin and are able to form tendon and enthesis-like tissues when implanted in vivo (Bi et al. 2007; Rui et al. 2010). Importantly, due to the absence of standardized protocols, the population of isolated tendon-resident stem and progenitor cells most likely varies due to differences in tendon type, donor age, and isolation protocol. Therefore, direct comparison of published results can be problematic. TSPCs have also been reported to express markers typical for embryonic stem cells (ESCs), such as Oct-4, Nanog, Sox2, c-myc, SSEA-4, and nucleostemin (Tan et al. 2013; Zhang and Wang 2010a), however fail to form teratomas (Tempfer H.; unpublished results). In vivo expression of these markers could only be detected at the tendon injury site, but not within intact tendons (Tan et al. 2013).

Taken together, our knowledge on the heterogeneous TSPC population is rather fragmentary. It is unclear if TSPCs represent a residual population of the embryonal tendon progenitors and the relationship of TSPCs to tenoblasts and tenocytes remains to be determined. This is mainly hampered by the lack of TSPC-specific markers, such as Tnmd, Scx, Mkx, and ColI, are expressed by both tenocytes and tendon stem and progenitor cells. Therefore, more studies tracking TSPCs in vivo and investigating their niche are imperative to make use of this cell source for treating injured tendons or other musculoskeletal disorders.

4 Tendon Stem Cell Niche

In human tendons, stem cells have been detected by immunohistochemical staining in the perivascular area expressing tendon (Scx, ColI, ColIII, Smad8) as well as stem/precursor cell (CD133, Musashi-1, Nestin, CD44, CD29) and pericyte-associated markers (αSMA) (Crisan et al. 2008; Tempfer et al. 2009). Some of these markers could also be identified in cells residing within the dense part of the tendon. Since TSPCs share many markers with mesenchymal stromal cells (MSCs), it is still not clear whether they are also pericytes as has been suggested by Caplan et al. for MSCs or whether they represent an own cell entity within the same perivascular niche (Caplan 2008; de Souza et al. 2016). This perivascular niche is defined by a variety of different factors providing signaling cues crucial for maintaining a balance of quiescence, self-renewal, and cell-fate commitment of the stem cell population residing in it. The stem cells are embedded within a very complex fibrous three-dimensional extracellular matrix consisting of a multitude of structural proteins such as collagens, glycoproteins, proteoglycans, and glycosaminoglycans. Besides the crosstalk with neighboring cells including tenocytes, pericytes, macrophages, mast, and endothelial cells via direct physical contact or paracrine signaling also soluble factors from the blood such as growth factors, cytokines, and oxygen contribute to establishing the TSPC-niche (see Fig. 2). Anchorage of the cells to the extracellular matrix via integrins, gap junction, and cadherin-based connections between tenocytes allow transmission of mechanical stimuli of the extracellular matrix to the cells eliciting respective signals which instruct TSPCs to either maintain their stem cell nature or direct them to differentiate into tenocytes or nontenocytes (McNeilly et al. 1996; Popov et al. 2015a; Schiele et al. 2013; Schwartz 2010; Stanley et al. 2007). Perturbing a single factor within this intricate network might therefore be sufficient to disturb/perturb tissue homeostasis.

4.1 Extracellular Matrix within the Niche

Regarding the influence of the extracellular matrix on stem cell biology not only the matrix composition is of relevance, but also its topography, nanostructure, stiffness/tissue elastic modulus, and strength (Ahmed and Ffrench-Constant 2016; Das and Zouani 2014; Tsimbouri 2015). Experiments using scaffolds with disoriented fiber

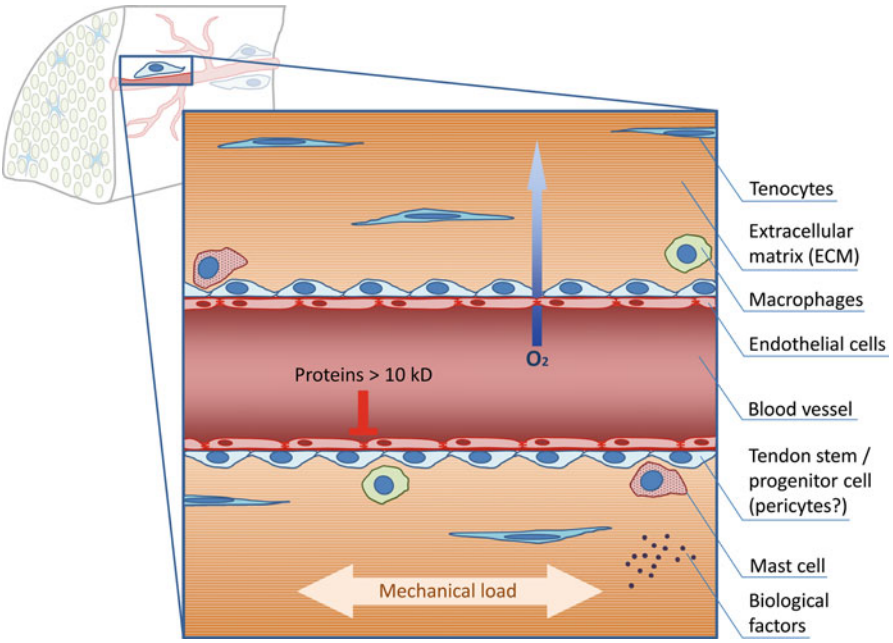


Fig. 2 Perivascular tendon stem and progenitor cell niche. The exact location of the tendon stem cell niche and the cellular and acellular components has not been fully defined yet. Tendon blood vessels are lined by endothelial cells forming a size selective barrier, most likely controlling the passage of blood-borne products into the tendon proper. The perivascular stem cells themselves most likely receive input from soluble factors, the extracellular matrix, neural inputs (?), the vascular network, and other cells (e.g., mast cells). Further, they perceive topographical information, such as ECM alignment and mechanical stress

alignment revealed that seeded stem cells differentiated into the osteogenic lineage, whereas parallel aligned scaffolds promoted tenogenic differentiation (Yin et al. 2010b). Interestingly, it has been shown that the fiber diameter had an even more significant effect on cellular behavior than fiber alignment, a fiber diameter of >2 μm appearing more suitable for tenogenic differentiation (Cardwell et al. 2014). Also, matrix stiffness seems to play an important role for stem cell fate, the stiffness of the ECM which favors tenogenic differentiation lying between values inducing myogenic and osteogenic differentiation (Das and Zouani 2014). By using polyacrylamide substrates functionalized with either fibronectin, collagen, or a combination of both and approximating the elastic modulus of tendon granulation tissue and the osteoid of healing bone with values ranging between 10 and 90 kPa, Sharma and Snedeker observed that seeded bone marrow stromal cells differentiated into the osteogenic lineage at a rigidity of around 80 kPa. In contrast, tenogenic differentiation occurred only on collagen substrate under moderate rigidity conditions (~30–50 kPa) (Sharma and Snedeker 2012).

The impact of the composition of the ECM has impressively been demonstrated by Bi et al. who by using $Bgn^{-/0}$ $Fmod^{-/-}$ knock out animals identified biglycan and

fibromodulin as two critical components that organize the niche (Bi et al. 2007). Mice deficient of these two matrix proteins showed impaired tendon formation and their tendons displayed ectopic ossification due to a change of TSPC fate from tenogenesis to osteogenesis. The authors speculate that changes in TSPC niche-associated ECM composition may perturb the balance of certain cytokines and growth factors stored within the EMC, which could be responsible for the altered TSPC cell fate.

Also, degradation of the ECM proteins by metalloproteinases known to be secreted upon extensive loading or progressive aging may liberate bound growth factors (e.g., VEGF, TGFß1), thereby modulating their bioactivities, and thus impact upon stem cell fate regulation (Koskinen et al. 2004; Spiesz et al. 2015).

4.2 Biomechanical and Biochemical Inputs Driving TSPC Fate

Mechanical loading, being an inherent part of the tendon environment, likely functions as a niche factor regulating the fate of TSPCs. Maintaining the pool of TSPCs and increasing the number of tenocytes from stretching-induced TSPC differentiation are two mechanisms that together provide an effective way for the maintenance of tendon homeostasis. Mechanical stimulation has been shown to upregulate Scx expression under physiological loading both in vitro and in vivo, suggesting that it might be required for tissue homeostasis (Maeda et al. 2011). Along these lines, 3 weeks of treadmill running for 50 min/day, 5 days a week, nearly doubled the proliferation rate of mouse Achilles and patellar TSPCs (Mendias et al. 2012; Zhang et al. 2010a).

There are several studies indicating that mechanical stimulation regulates stem cell proliferation and differentiation in a stretching magnitude-depending manner (Zhang and Wang 2010b). Although there is consensus about the fact that mechanical stimulation does have an effect on tenogenic differentiation, conflicting data exist regarding the extent of strain needed to be applied to be beneficial. While moderate running led to an upregulation of tenocyte-related genes, intensive running induced an increase in both tenocyte and nontenocyte-related genes (Zhang et al. 2010a). Whereas Zhang et al. report that in vitro low mechanical stretching (4%) increased the expression of only the tenocyte-related genes, and high mechanical stretching (8%) increased the expression of both tenocyte and nontenocyte-related genes, Rui et al. demonstrated that repetitive tensile loading at already 4% strain induced osteogenic differentiation (Rui et al. 2013b; Zhang and Wang 2013a). These differences observed might be due to differences in species used, duration, strain, and frequency of mechanical stretching that has been applied. As to the type of force acting on TSPCs, it is still not known whether increased tensile strain or compressive strain induces differentiation of TSPCs into nontenocytes (Lui and Chan 2011).

There are many studies investigating the effect of growth and differentiation factors such as GDF-5 (BMP 14), GDF-6 (BMP 13), GDF-7 (BMP 12), and insulin on mesenchymal stem cells demonstrating their potency to drive stem cell differentiation towards the tenogenic lineage (Hankemeier et al. 2005; Helm et al. 2001; Mazzocca et al. 2011; Park et al. 2010; Sassoon et al. 2012; Violini et al. 2009). Assuming that TSPCs represent specialized MSCs (or pericytes?) displaying a

tissue-specific phenotype, it is likely that TSPCs behave in a similar manner when exposed to the growth factors mentioned, but there are only few studies demonstrating this directly (Tokunaga et al. 2015). Incubation of rat patellar TSPCs with BMP-2 promoted glycosaminoglycan deposition, aggrecan expression, and enhanced nontenocyte differentiation of TSPCs (Rui et al. 2013b). Treatment of three clonal tendon cell lines with bFGF and TGFß significantly enhanced their proliferation (Salingcarnboriboon et al. 2003). Treatment of rat TSPCs with GDF-5 or connective tissue growth factor reduced differentiation along adipogenic and chondrogenic pathways and showed significantly enhanced tenogenic differentiation (Holladay et al. 2016; Lee et al. 2015; Ni et al. 2013). Moreover, IGF-1 treatment at 10 or 100 ng/ml maintained TSPC multipotency and phenotype (Holladay et al. 2016).

As for many tissues, in healthy human tendons macrophages and mast cells are localized in close proximity to blood vessels (Dakin et al. 2015). Whether these immune cells contribute to tendon homeostasis under physiological conditions or whether they solely serve as cellular guards monitoring their environment for stress signals is not known. Under inflammatory conditions or upon excessive loading, mast cells and macrophages become activated and secrete cytokines and inflammatory mediators such as prostaglandin E2 (PGE2). These cytokines lead to the recruitment of additional immune cells including leukocytes which in turn propagate cytokine production.

TSPCs have been shown to be affected by inflammatory mediators and biomechanical stress, driving them down paths of adipogenic and osteogenic differentiation. Prostaglandin E2, a major inflammatory mediator in tendons, decreased TSPC proliferation in vitro and induced both adipogenesis and osteogenesis of TSPCs in a dose-dependent manner (Zhang and Wang 2010c; Zhang and Wang 2012). Examination of the effects of IL-1β on the function of TSPC isolated from mouse injured Achilles tendons revealed that IL-1 β strongly reduced expression of tendon cell markers such as scleraxis and tenomodulin and irreversibly inhibited tenogenic differentiation (Zhang et al. 2015). Recently, Zhang and colleagues showed that leukocyte-enriched platelet-rich plasma (L-PRP) significantly reduced the proliferation of TSPCs in a concentration-dependent manner. Moreover, TSPCs grown in L-PRP differentiated into nontenocytes and produced more inflammatory factors such as membrane-associated prostaglandin synthase (mPGES) and IL-1β (Zhang et al. 2016).

Overall, our understanding of the various mechanical and biochemical stimuli driving and maintaining the tenogenic lineage in health and disease remains very limited, leaving ample room for future research.

4.3 Oxygen Tension

Oxygen tension has been identified as an important factor for maintaining stem cell stemness. The actual oxygen tension in tendons has not been unequivocally determined, but in most tissues it is estimated to be about 10% under physiological conditions; the pO_2 of human blood normally ranging between 75 and 100 mmHg, which is equivalent to O_2 gas levels of 10–13% (Holzwarth et al. 2010). Given that there are TSPCs residing in close proximity to the blood vessels and stem cells

located between aligned collagen fibers in some distance to the vasculature, it can be assumed that these two populations encounter different O_2 tensions, which might have an impact on their behavior. Lee et al. showed that culture of TSPCs under 2% O_2 tension increased cell number, colony number, and mRNA expression of tendon-related markers but reduced the osteogenic, adipogenic, and chondrogenic differentiation potentials (Lee et al. 2012). In line with these findings, it was shown that the culture of porcine tenocytes under hypoxic conditions significantly enhanced their expansion capacity (Zhang et al. 2010b). The important role of hypoxia is further underscored by the observation that human TSPCs maintain their stemness under hypoxic culture conditions by upregulating stem cell markers such as nucleostemin, Oct-4, Nanog, and SSEA-4 (Zhang and Wang 2013b).

4.4 Blood-Tendon Barrier

The recent description of a structural barrier formed by the endothelium lining blood vessels residing in tendons, which size-selectively controls the paracellular passage of macromolecules from the blood stream into the tendon proper, adds a new component to the network of structures potentially forming the tendon stem cell niche (Lehner et al. 2016). Experiments using dextran-labeled tracers of different size revealed that molecules larger than 10 kD are retained within the blood vessels. In vitro differentiation experiments using TSPCs further revealed that the presence of 10% serum in the differentiation-inducing medium promoted the formation of lipid droplets and increased the number of calcium deposits compared to cells cultured under serum-free adipogenic and osteogenic conditions. Further, in mature, healthy tendons very little turnover and thus proliferation occurs (Heinemeier et al. 2013). Upon injury or in tendinopathic tendons, going along with ruptured or leaky blood vessels, proliferation strongly increases most likely due to contact with serum components (e.g., growth factors), which under physiological conditions is restricted (Rolf et al. 2001). Further, serum-derived components potentially also drive the erroneous differentiation of TSPCs in situ, promoting tendon degeneration (see Fig. 3).

5 Tendon Development and Maturation

Tendon healing upon injury or long-term degeneration is a slow process which usually fails to restore tendon quality to the original status before injury. A concept gaining more and more acceptance is that successful interventions to improve (tendon) regeneration will have to recapitulate the developmental events that establish the native structure (Thomopoulos et al. 2010; Yang et al. 2013). Therefore, understanding the fundamental processes involved in embryonic tendon formation and identifying factors controlling these is a prerequisite for the development of novel and innovative treatment strategies. Similarly to most tissues and organs of the body, tendons do not develop independently from their surroundings, but they

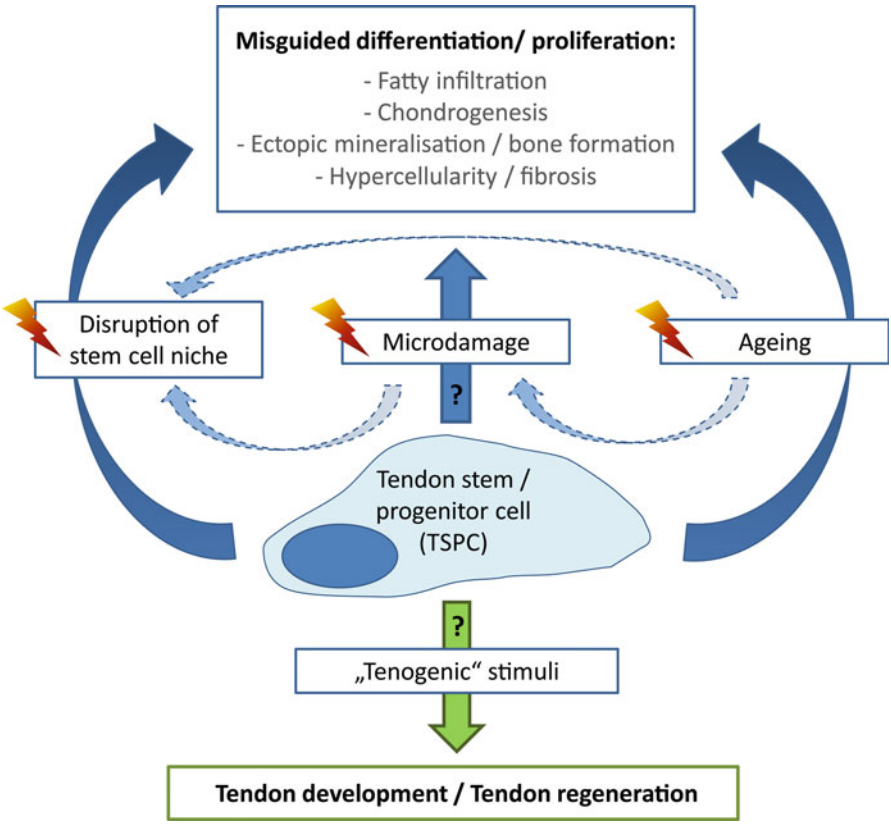


Fig. 3 Tendon stem/progenitor cells (TSPC) are suspected to participate in degenerative processes in aging or upon damage, due to their capacity to both proliferate and differentiate. The disruption of the stem cell niche in aged or damaged tendon is a key event in these unwanted processes. If and how TSPCs participate in tendon development and/or regeneration is still unclear, so are potential physiological tenogenic stimuli driving the maturation of tendon cells

strongly depend on signals from their neighboring tissues, mainly muscle and cartilage or bone (Deries and Thorsteinsdottir 2016; Kardon 1998).

As mentioned before, molecular characterization of tendon cells and/or tendon stem and progenitor cells is challenging and still a matter of debate (Lui 2013). As the master regulator gene(s) of the tendon lineage remain(s) to be identified, the intrinsic and extrinsic programs driving tenogenesis in vertebrates are yet to be discovered.

The identification of the basic helix-loop-helix transcription factor Scleraxis (Scx) as a crucial player in tendon development in 2001 (Schweitzer et al. 2001) was a major leap forward in understanding tendon development and significantly contributed to our current knowledge on early tendon formation. During early somite development, cartilage and muscle emerge from the myotome and sclerotome, responding to signals from surrounding tissues. The axial tendon lineage is established within the dorsolateral sclerotome as the somite matures, adjacent to

and beneath the myotome (Brent et al. 2005). This somatic compartment has also been termed syndetome (Brent et al. 2003). In contrast, craniofacial tendons have been shown to originate from the mesectoderm in mouse, chick, and zebrafish (Chen and Galloway 2014; Grenier et al. 2009).

The molecular program driving tendon development also depends on the anatomical location within the tendon and is influenced by the close association with muscle (myotendinous junction) or cartilage and bone (entheses). At the axial level, muscle is required for the initiation of tendon development (Brent et al. 2005), whereas for craniofacial and limb tendons muscles are not required for early initiation, but maintenance of tendons by inducing Scx expression (reviewed in (Gaut and Duprez 2016). Similarly, the formation of the bone-tendon unit requires complex signaling mechanisms. Zelzer E and Blitz E. et al. propose a so-called "segregation model," according to which a common multipotent "tenochondral progenitor," which is positive for both Scx and the chondrocyte associated transcription factor SRY (Sex Determining Region Y)-Box 9 (Sox9), gives rise to both cartilaginous bone primordia and early tendons (Zelzer et al. 2014). Interestingly, depletion of Sox9 in mice leads to a complete lack of cartilage, and tendon development, however, remains unaffected, indicating that Sox9 does not have a functional role in tendon cell differentiation (Blitz et al. 2013; Sugimoto et al. 2013).

Finally, mechanical load is also a crucial factor for tendon formation. Both FGF/ERK/MAPK and TGF-ß/SMAD2/3 signaling independently induce tendon formation downstream of mechanical loading, as it was shown in a chick embryo model (Havis et al. 2016).

5.1 Genes Involved in Tendon Development

So far, only two DNA-binding proteins strongly influencing tendon formation have been described: Scleraxis was identified to be a crucial factor in early development, regulating tendon progenitor cell fate (Brent et al. 2005; Schweitzer et al. 2001). Scleraxis is fundamental for tendon formation as depletion of this gene results in developmental tendon abnormalities varying between different tendons. However, it does not lead to complete tendon loss. Particularly muscle anchoring tendons remain largely unaffected, underlining the heterogeneity of tendon tissue development (Murchison et al. 2007).

The other transcription factor involved in tendon formation is the IRX family-related homeobox protein Mohawk (Mkx). It is expressed in various musculoskeletal progenitors and is required for neural crest cell migration during development (Chuang et al. 2010). Depletion of Mkx in mice leads to hypoplastic tendons throughout the body. Despite the reduction in tendon mass, the cell number in tail tendon fiber bundles remains similar between wild type and Mkx−/− mice. Again, no complete loss of tendons has been observed in Mkx−/− mice (Ito et al. 2010; Kimura et al. 2011).

Next to vertebrates, also invertebrates such as Drosophila possess tendons, of course displaying major differences, i.e., vertebrate tendons originating from the

mesoderm, whereas fly-tendons are ectodermal derivatives. In Drosophila tendon precursor are characterized by the expression of the transcription factor Stripe, an Egr (early growth response)-like transcription factor. Analysis of loss- and gain-of-function Stripe mutant phenotypes shows that Stripe is a key regulator of tendon cell specification and differentiation in fruit flies. (Frommer et al. 1996; Vorbruggen and Jackle 1997). The vertebrate homologues of Stripe, Early Growth response 1 and 2 (Egr1 and 2) are also involved in vertebrate tendon development: Adult tendons of Egr1−/− mice displayed a deficiency in the expression of tendon genes, including Scx, Col1a1, and Col1a2, and were mechanically weaker compared to their wildtype littermates. However, the observed effects are not severe enough to conclude that these genes are key drivers in vertebrate tendon cell differentiation. Nevertheless, the loss of Egr1 impairs tendon regeneration following injury in rodents and is thus considered a potential target to improve regeneration (Guerquin et al. 2013; Lejard et al. 2011).

Finally, tenomodulin (Tnmd) is one of the most common tendon-related marker proteins used for characterization (Dex et al. 2016) and its expression is closely associated with tendon differentiation during chick development. Loss of Tnmd in mice does not result in an embryonic phenotype, besides a modest decrease in tenocyte proliferation around birth and slightly altered collagen fibril size. However, at 6 months of age, no severe effects are obvious by depleting Tnmd (Docheva et al. 2005).

Taken together, many questions regarding tendon development remain unanswered and the underlying complex temporospatial expression patters ultimately resulting in the formation of mature tendons need to be unraveled to provide a basis for the effective treatment of tendon injury.

5.2 Vasculature and Cell Density in Developing Tendon

The formation of a vascular bed is a crucial factor for the development of virtually every tissue. Also under the aspect of blood vessels supplying a niche for stem and progenitor cells, vascularization during development is highly relevant (see Fig. 4). Unfortunately, for tendons the role of vascularization in development and maturation is still poorly defined (Tempfer and Traweger 2015). Peacock (1959) described embryonic tendons to be "supplied with a rich capillary network," based on the analysis of sections prepared from an 8-month-old human embryo (Peacock 1959). A study in postnatal, immature sheep describes a massive decline in both cellularity and vessel density in the tendon of the extrinsic flexor muscles of the fingers (musculus flexor digitorum superficialis), the meniscus and the cruciate ligaments between 1 and 40 weeks postnatally, with the strongest reduction occurring within the first 8 weeks. Remarkably, also the expression of vascular endothelial growth factor (VEGF) and of smooth muscle actin massively declines in the cells residing in the dense, collagenous tissue (Meller et al. 2009). In line with these findings, several studies point out the (relative) decrease of cell density during tendon maturation (Ippolito et al. 1980; Oryan and Shoushtari 2008). Given the fact that very little turnover of the extracellular matrix occurs in human tendons after termination of linear growth after ~17–18 years of age, low vascular supply seems appropriate (Heinemeier et al. 2013). However, in acute or

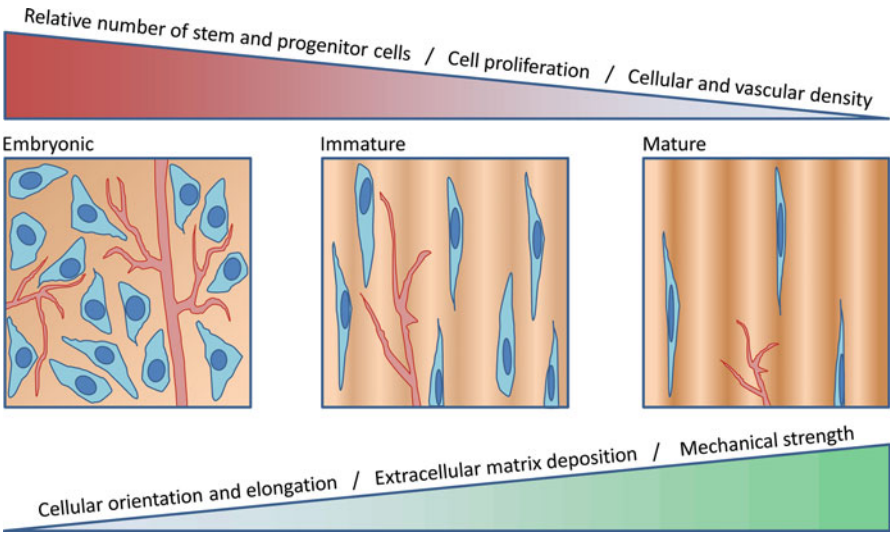


Fig. 4 The transition from embryonic to mature tendon is hallmarked by a decrease in cell density, a decline in the relative number of stem/progenitor cells, and massively reduced density of blood vessels. During this process, the amount of deposited extracellular matrix, the degree of cell alignment, and the mechanical strength of the tissue increase

chronic tendon injuries, hypervascularity does not seem to support functional recovery of tendons. Therefore, scarless tendon regeneration potentially requires a balanced manipulation of the angiogenic response.

6 Tendon Aging

With aging of tendons to withstand force declines and age-related tendon injuries are thought to be a result of changes in structure and mechanical properties of tendons, thus leading to physical frailty, reduced activity in the elderly, and a loss in general quality of life. Additionally, aged tendons only poorly respond to classical treatment strategies. The decline in functional integrity is based on changes to structural and mechanical properties of tendons. Furthermore, tendon-resident cells show qualitative and quantitative alterations dependent on the age of the tendon. The most characteristic age-related degenerations seen on the histological level are morphological changes of tendon cells, an altered collagen fiber structure, as well as accumulations of lipids and calcium depositions (Kannus et al. 2005). Together, these changes promote microdamages in the ECM, ultimately weakening tendons and increasing the risk for overuse injuries and full-thickness ruptures (Oliva et al. 2012).

Increasing age is related to a number of tendinopathies; a recent systematic review on rotator cuff degeneration revealed a prevalence of about 10% in patients aged 20 years or younger, increasing up to 40% in patients older than 60 years and up to 62% in patients 80 years and older (Teunis et al. 2014). Overall, the mechanisms

responsible for the functional decline in aged tendons are poorly described. Most studies on human tendon aging have been published based on degenerated tendon samples, revealing most likely changes due to degenerative processes (tendinopathy, tendinosis, ruptured tendons). However, virtually no data are available for healthy-aged tendons. A recent study demonstrated an age-related decrease of Secreted protein acidic and rich in cysteine (Sparc) in healthy-aged tendons. In vitro studies suggested that Sparc modulates the cell-ECM interaction of tendon-resident stem and progenitor cells and together with a change in ECM properties potentially impacts upon adipocyte differentiation as in aged and Sparc knockout tendons the accretion of lipids was observed (Gehwolf et al. 2016). Therefore, Sparc seems to have a limiting effect on adipogenesis in tendons, and its decreased expression with age might be an underlying cause for the formation of fatty depositions in aged tendons, ultimately increasing the risk of tendon degeneration and/or rupture.

Another common age-related phenomenon in tendons is ectopic mineralization, characterized by inappropriate depositions of calcium hydroxyapatite (De Vilder and Vanakker 2015; Kannus et al. 2005), frequently resulting in painful calcific tendinopathies (e.g., at the rotator cuff). The calcium deposits lead to changes in the mechanical properties of tendons and mineralized deposits often correlate with sites of rupture (Grases et al. 2015). However, it remains largely unclear how this mineralization occurs and whether some tendons are more affected than others. Alterations in the ECM composition, e.g., in the biglycan and decorin content, were described to promote ectopic ossification (Ameye et al. 2002). This is further supported by the reports of Bi et al. (2007) and Rui et al. (2013b) showing that the reduction in these small leucine-rich proteoglycans (SLRPs) at the tendon stem cell niche results in an aberrant differentiation of TSPCs, promoting degenerative calcification processes.

6.1 Cellular Senescence, Aging Tendon Stem Cells

Several studies have demonstrated that tendon resident stem and progenitor cells show age-related changes in vitro, including a reduced proliferation rate, lower colony-forming capacity, a diminished self-renewal capacity, and altered cell fate patterns. Generally, the number of tenocytes and TSPCs is reduced in aged tendons in vivo (see Fig. 4) and the progenitor cells show a higher tendency to differentiate towards the adipogenic lineage, which is supported by an increased expression of adipogenic marker genes such as Pparγ, Cebpα, perilipin, and leptin (Gehwolf et al. 2016; Zhou et al. 2010).

A study of Kohler et al. (2013) on cellular and molecular changes on human tendon progenitor cells derived from young-healthy and aged human Achilles tendons, however mostly displaying signs of degeneration, revealed that genes associated with cell adhesion, migration, or actin cytoskeleton, were significantly changed in their expression, resulting in dysregulated cell-matrix interactions. They provide evidence that the ROCK kinase pathway signaling is involved in tendon stem/progenitor cell aging/senescence (Kohler et al. 2013). The same group showed that the loss of tenomodulin (Tnmd) results in a reduced self-renewal and augmented

cell senescence of tendon progenitor cells (Alberton et al. 2015). Finally, it was also demonstrated that the downregulation of ephrin receptors 4, B2, and B4 (Eph4, EphB2, EphB4) in aged tendon cells limits the establishment of TSPC cell-cell interactions (Alberton et al. 2015).

Along the same lines, tendon-derived cells from healthy-aged mouse Achilles tendons showed age-related changes in cell morphology and their competence to contract matrices in 2D and 3D cell culture assays. Further, they were less spread on collagen type I-coated surfaces, formed larger focal adhesion complexes, and were characterized by an increase in the expression of paxillin when compared to young tendon cells. Additionally, a strong cortical cytoskeletal rearrangement was observed for healthy-aged tendon cells (Gehwolf et al. 2016).

6.2 Tendon Matrix Composition, Remodeling, Glycation, Collagen Turnover

As described in more detail further above, the tendon extracellular matrix is predominantly composed of hierarchically arranged collagen type I fibers. The ECM undergoes age-related changes in structure, composition and organization, and protein turnover. A well-accepted method assessing aging on the protein level is the analysis of amino acid racemization. Amino acids are incorporated in the tissue in their L-form and with time undergo racemization to their D-form. The most rapidly converting amino acid is aspartic acid, which is easily detectable in aged biological tissues, and therefore, increased levels of D-Asp are an indicator for a reduced/slower protein turnover. Together with the determination of collagen degradation, amino acid racemization is a valuable tool to analyze matrix aging and turnover. The reduced matrix turnover rate indicates a decreased ability/activity of tenocytes to repair microdamages potentially leading to their accumulation with age.

On the ultrastructural level, in aged C57BL/6 mouse tendons, the collagen fibril diameters are increased and fibrils are more densely packed (Gehwolf et al. 2016; Goh et al. 2008, 2012). Further, the fibers are less well oriented/aligned in old mouse tendons (Dunkman et al. 2013; Gehwolf et al. 2016) and variations in collagen crimping have been reported (Gautieri et al. 2016; Legerlotz et al. 2014). Also, a reduction in extracellular water content (Kannus et al. 2005), the reduced expression of collagen type I and of several SLRPs (e.g., decorin, biglycan, fibromodulin) and glycoproteins (Dunkman et al. 2014; Dunkman et al. 2013; Gehwolf et al. 2016), a higher collagen crosslinking rate, and the accumulation of advanced glycation end-products (Snedeker and Gautieri 2014) are typically seen for aged tendons. Interestingly, decorin seems to promote age-related changes in collagen fibril maturation and biomechanics, as in decorin knock out mice these effects were ameliorated (Dunkman et al. 2013).

Finally, several studies demonstrated an increase in tendon matrix degrading enzymes with age. Next to matrix-metalloproteinase-1 (MMP-1) (Riley et al. 2002), an enhanced activity of MMP-2 and MMP-9 has been associated with tendon aging (Dudhia et al. 2007; Yu et al. 2013).

Table 1 Cellular and extracellular changes in aged tendons

cellular level	cell number ↓	(Birch et al., 1999; Gehwolf et al., 2016)
	DNA content ↓	(Birch et al., 1999)
	stem/progenitor cell number ↓	(Zhou et al., 2010)
	self-renewal capacity ↓	(Popov et al., 2015b; Zhou et al., 2010)
	colony forming capacity ↓	(Kohler et al., 2013; Zhou et al., 2010)
	Tenomodulin expression ↓	(Alberton et al., 2015)
	Scleraxis expression ↓	(Zhou et al., 2010)
	inflammatory response ↓	(Dakin et al., 2012; Humbert et al., 1976; Kietrys et al., 2012; Speed, 2016)
	metabolic activity ↓	(Almekinders and Deol, 1999; Floridi et al., 1981)
	proliferation ↓	(Kohler et al., 2013; Tsai et al., 2011)
	cell-cell interactions ↓	(Alberton et al., 2015)
	adipogenic differentiation ↑	(Gehwolf et al., 2016; Zhou et al., 2010)
	inflammatory processes ↑	(Dakin et al., 2012; Speed, 2016)
	senescence ↑	(Kohler et al., 2013; Popov et al., 2015b)
extracellular matrix	total collagen content ↔	(Birch et al., 2016; Gehwolf et al., 2016)
	fibre alignment ↓	(Dunkman et al., 2013; Gehwolf et al., 2016)
	crimp angle ↓	(Legerlotz et al., 2014)
	collagen turnover ↓	(Birch et al., 1999; Birch et al., 2016)
	lipid accumulation ↑	(Gehwolf et al., 2016; Kannus et al., 2005)
	collagen type 3 ↑	(Birch et al., 1999; Birch et al., 2016; Gehwolf et al., 2016)
	collagen fibril diameter ↑	(Dunkman et al., 2013; Gehwolf et al., 2016; Goh et al., 2012)
	collagen crosslinking ↑	(Bank et al., 1999)
	collagen degradation ↑	(Birch et al., 2016; Thorpe et al., 2010)
	collagen glycosylation ↑	(Gautieri et al., 2016; Snedeker and Gautieri, 2014)
	ectopic mineralization ↑	(Bi et al., 2007; Rui et al., 2011)
	MMP-1, -2, -9 activity ↑	(Dudhia et al., 2007; Riley et al., 2002)

In summary, these changes in tendon matrix and the associated biomechanical changes underlie the increased risk of tendon injury with age (see also Table 1).

6.3 Mechanical Properties

The mechanical properties of tendons depend on their anatomical location, load, training, and structural characteristics. Material properties and structure of tendons depend mainly on the anisotropic organization of the ECM which improve from birth to maturity and deteriorate with age (Wang et al. 2012). However, published results on the mechanics of aged tendons are partially contradictory; some studies show that aged tendons display a higher tensile strain (Onambele et al. 2006), whereas others report a decreased tensile strain (Kubo et al. 2007), or no impact on most tendon mechanical properties with age (Arampatzis et al. 2007). Similarly discrepant results have been published for tendon stiffness (Flahiff et al. 1995; Gehwolf et al. 2016; Lewis and Shaw 1997; Stenroth et al. 2012; Wu et al. 2015). However, the comparability of these results has to be questioned since different tendons from various species and fresh or embalmed tendon tissues were used for these studies. Furthermore, one must also consider whether altered mechanical properties of healthy-aged tendons are comparable to tendons undergoing

degenerative processes due to other extrinsic or intrinsic factors such as lifestyle, chronic diseases, a specific genetic background, or whether aging alone can account for these changes.

6.4 Tendon Aging and Inflammatory Processes

Cellular aging/senescence has also been linked to a decreased ability to manage inflammatory processes resulting in a chronic low-level inflammation, termed "inflammaging." These changes lead to an increase in the systemic pro-inflammatory status with a concomitantly elevated inflammatory cytokine production/secretion in aged tissues. As a consequence, organs and tissues are more susceptible to frailty and age-related diseases. The correlation of age and the increase in tendon pathologies and injury risk are well recognized. However, the ability of old tendons to cope with inflammation and the contribution of immune-senescence to the pathogenesis of tendon disorders in the elderly are poorly understood.

Dakin and colleagues described an age-related reduced expression of FPR2/ALX and a higher secretion of PGE2 in old tendons. Additionally, in tendons of old horses treated with IL-1ß, the expression of FPR2/ALX and PGE2 secretion, indicating "inflammaging" might be present in aging tendons and old tendons, have a reduced capacity to cope with inflammation. Further, recent studies demonstrated that inflammatory events affecting tendon homeostasis and healing may be responsible for an inappropriate function of the tendon, an increased risk of tendon rupture and re-ruptures (Morita et al. 2016). Along the same lines, healthy-aged mouse Achilles tendons displayed a differential expression of immune response-related genes (Gehwolf et al. 2016). Taken together, a decreased ability to manage inflammatory processes may contribute to the reduced tendon healing capacity in the elderly.

7 Tendon Regeneration: Current Challenges and Future Strategies

Unlike highly regenerative tissues, such as skin or bone, tendons do not functionally regenerate but merely heal by forming a scar tissue with inferior mechanical properties and an increased risk of (re)rupture. Generally, tendon ruptures are believed only to occur upon prior damage to the tissue, unless an acute laceration takes place. This predamage can have a variety of etiologies, such as microdamage due to overload, inflammation, tissue weakening due to systemic diseases such as diabetes or obesity, or as discussed previously due to age-related changes. However, in many cases the exact etiology remains unclear (Abate et al. 2009; Oliva et al. 2011). Many terms have been used to describe these disorders, including tendinitis, tendinosis, and paratenonitis. Commonly, they are described by their symptoms, which usually encompass pain, swelling, hypervascularization, deposition of calcific minerals, and long-term loss of fiber orientation. Likely, these symptoms are the results of a failed healing response (Longo et al. 2009). The role of inflammation in

these events is still a matter of debate; however, at least in the initial phase of disease, involvement of inflammatory cells seems likely (Abate et al. 2009; Dean et al. 2016). In order to circumvent the unclear and probably heterogeneous etiology, the umbrella term "tendinopathy" has been introduced (Maffulli et al. 1998).

The role of tendon stem progenitor cells (TSPC) in the progression of tendinopathy as well as their potential role in regeneration is currently under investigation. As mentioned above, these cells have the potential to differentiate into osteoblasts, chondrocytes, and adipocytes as well as into tendon cell-like cells (Bi et al. 2007; Rui et al. 2010). There is some evidence from in vitro experiments using human and rodent TSPC that cell fate, senescence, and clonogenicity are altered by aging and degeneration, leading to speculations that the TSPC pool is becoming exhausted in terms of size and functional fitness and potentially are the origin of various tendinopathies (Kohler et al. 2013; Rui et al. 2013a). However, to date there are no solid in vivo data available describing the fate of TSPCs during tendinopathies.

7.1 Cellular Mechanisms of Tendon Repair

The tendon repair process follows three distinct phases: (1) An early inflammatory phase, with macrophage invasion and/or proliferation. At this early phase, which lasts about 1 week, macrophages display a proinflammatory M1-polarization, secreting proinflammatory cytokines such as IL-1ß, IL-6 and TNF (Voleti et al. 2012). (2) In the second phase, fibroblasts proliferate within the wound area, a process guided by M2-polarized macrophages. At this stage, also erroneous differentiation of stem cells is potentially initiated (Runesson et al. 2015). (3) In the third phase, the so-called "remodeling phase," the fibroblasts begin to produce, deposit, orient, and crosslink fibrillar collagens. During this process, a disturbance of the fine-tuned balance between M1 and M2 macrophages may lead to improper tendon repair (Sugg et al. 2014). Therefore, guided immunomodulation to prevent unfavorable scar formation may be an interventional strategy to improve tendon healing. One option currently pursued to modulate the inflammatory response is the use of mesenchymal stem cells (see below).

The origin or source of cells involved in tendon repair remains poorly characterized. They potentially originate from blood, surrounding adipose tissue or epitenon sheaths (Dyment et al. 2013; Nourissat et al. 2015). It has also been proposed that TSPCs are activated after tendon injury. In a rat Achilles tenotomy model, an increased number of TSPC positive for the stem cell markers Oct3/4 and nucleostemin are found at early time points after injury. Their distribution becomes more distinct at later time points, with proteoglycan-rich areas containing clusters of more chondrocyte-like cells and containing higher proportions of nucleostemin-positive cells. This could be an indication of a stem cell-related coordination problem in the healing processes, which potentially has a negative impact on the mechanical function after an Achilles mid-tendon rupture (Runesson et al. 2015). In another study making use of a rat model of Achilles tendon injury, perivascular cells

were shown to contribute to tendon repair. These cells with neural crest cell-like characteristics, such as the expression of p75, vimentin, and Sox10, migrate from the vessel to the interstitial space upon injury, where they deposit extracellular matrix (Xu et al. 2015). However, if and to what extent the contribution of these cells during tissue repair is beneficial for tendon quality and their role in tendon development remains elusive.

7.2 Use of Stem Cells for Tendon Treatment

Tissue engineering involves the use of cells, biomaterials, growth factors, enzymatic antagonists, or a combination thereof with the aim of promoting tissue repair. Terminally differentiated cells as well as stem/progenitor cells have been used in tendon tissue engineering approaches. Among these different cell types, stem cells have attracted a great interest in tissue engineering as they can continuously reproduce themselves while maintaining the ability to differentiate into various cell types (Lui 2015). However, several factors need to be considered for choosing the ideal cell type for regenerative approaches: The cells need to be free of ethical concerns, safe in terms of tumor risk, must not cause adverse immunoreactions, must have the potential to functionally replace lost tendon cells, should be obtainable with minimal morbidity and pain for the patient/donor, and should have a high proliferation rate in vitro, in order to obtain a satisfying number of cells in a reasonable time span. Moreover, the cells should not undergo uncontrolled differentiation after implantation. As ectopic osteogenesis was frequently observed, following stem cell implantation to tendons remains a major concern (Harris et al. 2004). Another question related to the debate on the best cell source is whether autologous or allogeneic cells should be used. The main advantage of the latter is faster and more controlled availability, the main concern being their potential immunogenicity (Hilfiker et al. 2011).

Various cell types have been considered to augment tendon repair and have been evaluated in preclinical animal studies over the last two decades. Next to mesenchymal stromal cells of various tissue origin (e.g., bone marrow, adipose tissue), skin fibroblasts, embryonic stem cells, and induced pluripotent stem cells (iPSC) have been evaluated (Docheva et al. 2015; Gaspar et al. 2015; Yin et al. 2010a). In addition, the identification of tendon resident stem/progenitor cells (Bi et al. 2007; Salingcarnboriboon et al. 2003) set the stage for their application as a therapeutic tool to treat diseased tendons. In various small and large animal models, autologous TSCPs were tested for their potential to improve tendon quality. In a rabbit rotator cuff defect model, the authors concluded that the implantation of autologous tenocytes on collagen-based, biodegradable scaffolds resulted in improved rotator cuff tendon healing and remodeling when compared with implantation of the scaffold alone, suggesting their use might be beneficial to treat massive rotator cuff tears (Chen et al. 2007). Similarly, in a rabbit Achilles tendinopathy model, implanted TSPCs were found to have beneficial effects on tendon remodeling,

histological outcomes, collagen content, and tensile strength of the treated tendons (Chen et al. 2011).

Besides dermal fibroblasts, nonbulbar dermal sheath (NBDS) cells isolated from the hair follicle, and adipose-derived stem cells (Usuelli et al. 2017), TSPCs have also been applied in clinical trials to treat diseased tendons. A case series including 20 patients suffering from severe, chronic resistant lateral epicondylitis shows that the application of autologous, in vitro expanded tendon cells obtained from the patient's patella tendon is safe. Also MRI-scores significantly improved over a 12-month period after the injection (Wang et al. 2013). The same patient cohort was followed up to 5 years, showing the initial benefit was persisting (Wang et al. 2015). However, efficacy of this treatment remains to be confirmed in a randomized, controlled trial. A potential limitation of this approach is the relatively high donor site morbidity as punch biopsies taken from tendons such as the patella can cause tendinopathies themselves, even though no adverse events were reported in these studies.

Currently, the therapeutic use of vesicles released from cells, named extracellular vesicles or EVs, has gained tremendous attention (Camussi et al. 2010; Ratajczak et al. 2006). EVs are a heterogeneous population of small vesicles constituted by a circular fragment of membrane containing cytoplasm components which are released by different cell types. Extracellular vesicles derived from MSCs seem to mediate beneficial therapeutic effects in a variety of different diseases. Besides immunomodulation, also angiogenesis and tumor growth is modulated by EVs (Lener et al. 2015). The research on the mechanisms exerted by these vesicles and their potential clinical application is still an emerging field; however, a variety of clinical trials are already on their way, including treatment of dermal wounds, psoriasis, and type I diabetes. As EVs elicit several of the biological actions also observed for adult stem cells, they resemble an attractive option to treat diseased tendons, as they eliminate some of the potential disadvantages when using active, replicating cells that may undergo mal-differentiation or mutation (Tetta et al. 2012).

In summary, the discovery of a population of stem/progenitor cells in tendons has permitted a rapid progress in the study of tendon biology. However, the role of these cells in tendon development and homeostasis remains largely unclear. Also, our understanding of their role in tendon degeneration and aging is far from complete. Therefore, great efforts are required to establish a solid foundation for successfully targeting endogenous TSPCs to improve tendon repair or make use of these cells as a tissue engineering tool.

8 Conclusions

The current options for conservative or surgical treatment of tendon injuries often do not provide satisfactory, long-term outcomes. Therefore, new therapeutic approaches are needed to augment tendon repair, or even allow functional regeneration of tendon tissue. However, although quite some progress has been made in repairing tendon, the clinical impact remains limited. Also, research progress has been hampered by

our incomplete understanding of tendon development, physiology, and healing, mainly due to the absence of marker proteins specific for the tenogenic lineage. Next to the development of sophisticated scaffolds to support tendon repair, strategies employing cell and gene therapy and other biologicals (e.g., growth factors) are actively being pursued. Most likely a combination thereof will be required to achieve scarless healing and full restoration of the biomechanical properties of tendon tissue after injury or disease and rigorous scientific investigations and scrutiny will be required to translate their full therapeutic potential.

References

Abate M, Silbernagel KG, Siljeholm C, Di Iorio A, De Amicis D, Salini V, Werner S, Paganelli R (2009) Pathogenesis of tendinopathies: inflammation or degeneration? Arthritis Res Ther 11:235

Ahmed M, Ffrench-Constant C (2016) Extracellular matrix regulation of stem cell behavior. Curr Stem Cell Rep 2:197–206

Alberton P, Dex S, Popov C, Shukunami C, Schieker M, Docheva D (2015) Loss of tenomodulin results in reduced self-renewal and augmented senescence of tendon stem/progenitor cells. Stem Cells Dev 24:597–609

Almekinders LC, Deol G (1999) The effects of aging, antiinflammatory drugs, and ultrasound on the in vitro response of tendon tissue. Am J Sports Med 27:417–421

Ameye L, Aria D, Jepsen K, Oldberg A, Xu T, Young MF (2002) Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. FASEB J 16:673–680

Ansorge HL, Meng X, Zhang G, Veit G, Sun M, Klement JF, Beason DP, Soslowsky LJ, Koch M, Birk DE (2009) Type XIV collagen regulates Fibrillogenesis: PREMATURE COLLAGEN FIBRIL GROWTH AND TISSUE DYSFUNCTION IN NULL MICE. J Biol Chem 284:8427–8438

Arampatzis A, Karamanidis K, Morey-Klapsing G, De Monte G, Stafilidis S (2007) Mechanical properties of the triceps surae tendon and aponeurosis in relation to intensity of sport activity. J Biomech 40:1946–1952

Bailey AJ (2001) Molecular mechanisms of ageing in connective tissues. Mech Ageing Dev 122:735–755

Banes AJ, Donlon K, Link GW, Gillespie Y, Bevin AG, Peterson HD, Bynum D, Watts S, Dahners L (1988) Cell populations of tendon: a simplified method for isolation of synovial cells and internal fibroblasts: confirmation of origin and biologic properties. J Orthop Res 6:83–94

Bank RA, TeKoppele JM, Oostingh G, Hazleman BL, Riley GP (1999) Lysylhydroxylation and non-reducible crosslinking of human supraspinatus tendon collagen: changes with age and in chronic rotator cuff tendinitis. Ann Rheum Dis 58:35–41

Benjamin M, Ralphs JR (1997) Tendons and ligaments – an overview. Histol Histopathol 12:1135–1144

Benjamin M, Kaiser E, Milz S (2008) Structure-function relationships in tendons: a review. J Anat 212:211–228

Berenson MC, Blevins FT, Plaas AH, Vogel KG (1996) Proteoglycans of human rotator cuff tendons. J Orthop Res 14:518–525

Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, Li L, Leet AI, Seo BM, Zhang L, Shi S, Young MF (2007) Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med 13:1219–1227

Birch HL, Bailey JV, Bailey AJ, Goodship AE (1999) Age-related changes to the molecular and cellular components of equine flexor tendons. Equine Vet J 31:391–396

Birch HL, Peffers MJ, Clegg PD (2016) Influence of ageing on tendon homeostasis. Adv Exp Med Biol 920:247–260
Blitz E, Sharir A, Akiyama H, Zelzer E (2013) Tendon-bone attachment unit is formed modularly by a distinct pool of Scx- and Sox9-positive progenitors. Development 140:2680–2690
Brent AE, Schweitzer R, Tabin CJ (2003) A somitic compartment of tendon progenitors. Cell 113:235–248
Brent AE, Braun T, Tabin CJ (2005) Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. Development 132:515–528
Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L (2010) Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int 78:838–848
Canty EG, Kadler KE (2002) Collagen fibril biosynthesis in tendon: a review and recent insights. Comp Biochem Physiol A Mol Integr Physiol 133:979–985
Canty EG, Kadler KE (2005) Procollagen trafficking, processing and fibrillogenesis. J Cell Sci 118:1341–1353
Caplan AI (2008) All MSCs are pericytes? Cell Stem Cell 3:229–230
Cardwell RD, Dahlgren LA, Goldstein AS (2014) Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage. J Tissue Eng Regen Med 8:937–945
Carvalho HF, Felisbino SL, Covizi DZ, Della Colleta HH, Gomes L (2000) Structure and proteoglycan composition of specialized regions of the elastic tendon of the chicken wing. Cell Tissue Res 300:435–446
Chakravarti S (2002) Functions of lumican and fibromodulin: lessons from knockout mice. Glycoconj J 19:287–293
Chen JW, Galloway JL (2014) The development of zebrafish tendon and ligament progenitors. Development 141:2035–2045
Chen JM, Willers C, Xu J, Wang A, Zheng MH (2007) Autologous tenocyte therapy using porcine-derived bioscaffolds for massive rotator cuff defect in rabbits. Tissue Eng 13:1479–1491
Chen J, Yu Q, Wu B, Lin Z, Pavlos NJ, Xu J, Ouyang H, Wang A, Zheng MH (2011) Autologous tenocyte therapy for experimental Achilles tendinopathy in a rabbit model. Tissue Eng Part A 17:2037–2048
Chuang HN, Cheng HY, Hsiao KM, Lin CW, Lin ML, Pan H (2010) The zebrafish homeobox gene irxl1 is required for brain and pharyngeal arch morphogenesis. Dev Dyn 239:639–650
Chuen FS, Chuk CY, Ping WY, Nar WW, Kim HL, Ming CK (2004) Immunohistochemical characterization of cells in adult human patellar tendons. J Histochem Cytochem 52:1151–1157
Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313
Dakin SG, Dudhia J, Werling NJ, Werling D, Abayasekara DR, Smith RK (2012) Inflamm-aging and arachadonic acid metabolite differences with stage of tendon disease. PLoS One 7:e48978
Dakin SG, Martinez FO, Yapp C, Wells G, Oppermann U, Dean BJ, Smith RD, Wheway K, Watkins B, Roche L, Carr AJ (2015) Inflammation activation and resolution in human tendon disease. Sci Transl Med 7:311ra173
Das RK, Zouani OF (2014) A review of the effects of the cell environment physicochemical nanoarchitecture on stem cell commitment. Biomaterials 35:5278–5293
Davidson CJ, Ganion LR, Gehlsen GM, Verhoestra B, Roepke JE, Sevier TL (1997) Rat tendon morphologic and functional changes resulting from soft tissue mobilization. Med Sci Sports Exerc 29:313–319
de Souza LE, Malta TM, Kashima Haddad S, Covas DT (2016) Mesenchymal stem cells and Pericytes: to what extent are they related? Stem Cells Dev 25:1843
De Vilder EY, Vanakker OM (2015) From variome to phenome: pathogenesis, diagnosis and management of ectopic mineralization disorders. World J Clin Cases 3:556–574

Dean BJ, Gettings P, Dakin SG, Carr AJ (2016) Are inflammatory cells increased in painful human tendinopathy? A systematic review. Br J Sports Med 50:216–220

Deries M, Thorsteinsdottir S (2016) Axial and limb muscle development: dialogue with the neighbourhood. Cell Mol Life Sci 73:4415–4431

Dex S, Lin D, Shukunami C, Docheva D (2016) Tenogenic modulating insider factor: systematic assessment on the functions of tenomodulin gene. Gene 587:1–17

Docheva D, Hunziker EB, Fassler R, Brandau O (2005) Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol 25:699–705

Docheva D, Muller SA, Majewski M, Evans CH (2015) Biologics for tendon repair. Adv Drug Deliv Rev 84:222–239

Dudhia J, Scott CM, Draper ER, Heinegard D, Pitsillides AA, Smith RK (2007) Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. Aging Cell 6:547–556

Dunkman AA, Buckley MR, Mienaltowski MJ, Adams SM, Thomas SJ, Satchell L, Kumar A, Pathmanathan L, Beason DP, Iozzo RV, Birk DE, Soslowsky LJ (2013) Decorin expression is important for age-related changes in tendon structure and mechanical properties. Matrix Biol 32:3–13

Dunkman AA, Buckley MR, Mienaltowski MJ, Adams SM, Thomas SJ, Kumar A, Beason DP, Iozzo RV, Birk DE, Soslowsky LJ (2014) The injury response of aged tendons in the absence of biglycan and decorin. Matrix Biol 35:232–238

Dyment NA, Liu CF, Kazemi N, Aschbacher-Smith LE, Kenter K, Breidenbach AP, Shearn JT, Wylie C, Rowe DW, Butler DL (2013) The paratenon contributes to scleraxis-expressing cells during patellar tendon healing. PLoS One 8:e59944

Flahiff CM, Brooks AT, Hollis JM, Vander Schilden JL, Nicholas RW (1995) Biomechanical analysis of patellar tendon allografts as a function of donor age. Am J Sports Med 23:354–358

Floridi A, Ippolito E, Postacchini F (1981) Age-related changes in the metabolism of tendon cells. Connect Tissue Res 9:95–97

Frommer G, Vorbruggen G, Pasca G, Jackle H, Volk T (1996) Epidermal egr-like zinc finger protein of drosophila participates in myotube guidance. EMBO J 15:1642–1649

Gaspar D, Spanoudes K, Holladay C, Pandit A, Zeugolis D (2015) Progress in cell-based therapies for tendon repair. Adv Drug Deliv Rev 84:240–256

Gaut L, Duprez D (2016) Tendon development and diseases. Wiley Interdiscip Rev Dev Biol 5:5–23

Gautieri A, Passini FS, Silvan U, Guizar-Sicairos M, Carimati G, Volpi P, Moretti M, Schoenhuber H, Redaelli A, Berli M, Snedeker JG (2016) Advanced glycation end-products: mechanics of aged collagen from molecule to tissue. Matrix Biol. https://doi.org/10.1016/j.matbio.2016.09.001

Gehwolf R, Wagner A, Lehner C, Bradshaw AD, Scharler C, Niestrawska JA, Holzapfel GA, Bauer HC, Tempfer H, Traweger A (2016) Pleiotropic roles of the matricellular protein Sparc in tendon maturation and ageing. Sci Rep 6:32635

Goh KL, Holmes DF, Lu HY, Richardson S, Kadler KE, Purslow PP, Wess TJ (2008) Ageing changes in the tensile properties of tendons: influence of collagen fibril volume fraction. J Biomech Eng 130:021011

Goh KL, Holmes DF, Lu Y, Purslow PP, Kadler KE, Bechet D, Wess TJ (2012) Bimodal collagen fibril diameter distributions direct age-related variations in tendon resilience and resistance to rupture. J Appl Physiol (1985) 113:878–888

Gordon JA, Freedman BR, Zuskov A, Iozzo RV, Birk DE, Soslowsky LJ (2015) Achilles tendons from decorin- and biglycan-null mouse models have inferior mechanical and structural properties predicted by an image-based empirical damage model. J Biomech 48:2110–2115

Grases F, Muntaner-Gimbernat L, Vilchez-Mira M, Costa-Bauza A, Tur F, Prieto RM, Torrens-Mas M, Vega FG (2015) Characterization of deposits in patients with calcific tendinopathy of the supraspinatus. Role of phytate and osteopontin. J Orthop Res 33:475–482

Grenier J, Teillet MA, Grifone R, Kelly RG, Duprez D (2009) Relationship between neural crest cells and cranial mesoderm during head muscle development. PLoS One 4:e4381

Guerquin MJ, Charvet B, Nourissat G, Havis E, Ronsin O, Bonnin MA, Ruggiu M, Olivera-Martinez I, Robert N, Lu Y, Kadler KE, Baumberger T, Doursounian L, Berenbaum F, Duprez D (2013) Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. J Clin Invest 123:3564–3576

Hankemeier S, Keus M, Zeichen J, Jagodzinski M, Barkhausen T, Bosch U, Krettek C, Van Griensven M (2005) Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments. Tissue Eng 11:41–49

Harris MT, Butler DL, Boivin GP, Florer JB, Schantz EJ, Wenstrup RJ (2004) Mesenchymal stem cells used for rabbit tendon repair can form ectopic bone and express alkaline phosphatase activity in constructs. J Orthop Res 22:998–1003

Havis E, Bonnin MA, Esteves de Lima J, Charvet B, Milet C, Duprez D (2016) TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. Development 143:3839–3851

Heinemeier KM, Schjerling P, Heinemeier J, Magnusson SP, Kjaer M (2013) Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb (14)C. FASEB J 27:2074–2079

Helm GA, Li JZ, Alden TD, Hudson SB, Beres EJ, Cunningham M, Mikkelsen MM, Pittman DD, Kerns KM, Kallmes DF (2001) A light and electron microscopic study of ectopic tendon and ligament formation induced by bone morphogenetic protein-13 adenoviral gene therapy. J Neurosurg 95:298–307

Hilfiker A, Kasper C, Hass R, Haverich A (2011) Mesenchymal stem cells and progenitor cells in connective tissue engineering and regenerative medicine: is there a future for transplantation? Langenbecks Arch Surg 396:489–497

Holladay C, Abbah SA, O'Dowd C, Pandit A, Zeugolis DI (2016) Preferential tendon stem cell response to growth factor supplementation. J Tissue Eng Regen Med 10:783–798

Holzwarth C, Vaegler M, Gieseke F, Pfister SM, Handgretinger R, Kerst G, Muller I (2010) Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells. BMC Cell Biol 11:11

Humbert F, Montesano R, Perrelet A, Orci L (1976) Junctions in developing human and rat kidney: a freeze-fracture study. J Ultrastruct Res 56:202–214

Ippolito E, Natali PG, Postacchini F, Accinni L, De Martino C (1980) Morphological, immunochemical, and biochemical study of rabbit achilles tendon at various ages. J Bone Joint Surg Am 62:583–598

Ito Y, Toriuchi N, Yoshitaka T, Ueno-Kudoh H, Sato T, Yokoyama S, Nishida K, Akimoto T, Takahashi M, Miyaki S, Asahara H (2010) The Mohawk homeobox gene is a critical regulator of tendon differentiation. Proc Natl Acad Sci U S A 107:10538–10542

Kadler KE, Hojima Y, Prockop DJ (1990) Collagen fibrils in vitro grow from pointed tips in the C- to N-terminal direction. Biochem J 268:339–343

Kalamajski S, Liu C, Tillgren V, Rubin K, Oldberg A, Rai J, Weis M, Eyre DR (2014) Increased C-telopeptide cross-linking of tendon type I collagen in fibromodulin-deficient mice. J Biol Chem 289:18873–18879

Kannus P (2000) Structure of the tendon connective tissue. Scand J Med Sci Sports 10:312–320

Kannus P, Paavola M, Józsa L (2005) Aging and degeneration of tendons. Springer, London

Kardon G (1998) Muscle and tendon morphogenesis in the avian hind limb. Development 125:4019–4032

Kielty CM, Sherratt MJ, Shuttleworth CA (2002) Elastic fibres. J Cell Sci 115:2817–2828

Kietrys DM, Barr-Gillespie AE, Amin M, Wade CK, Popoff SN, Barbe MF (2012) Aging contributes to inflammation in upper extremity tendons and declines in forelimb agility in a rat model of upper extremity overuse. PLoS One 7:e46954

Kimura W, Machii M, Xue X, Sultana N, Hikosaka K, Sharkar MT, Uezato T, Matsuda M, Koseki H, Miura N (2011) Irxl1 mutant mice show reduced tendon differentiation and no patterning defects in musculoskeletal system development. Genesis 49:2–9

Kohler J, Popov C, Klotz B, Alberton P, Prall WC, Haasters F, Muller-Deubert S, Ebert R, Klein-Hitpass L, Jakob F, Schieker M, Docheva D (2013) Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration. Aging Cell 12:988–999

Koskinen SO, Heinemeier KM, Olesen JL, Langberg H, Kjaer M (2004) Physical exercise can influence local levels of matrix metalloproteinases and their inhibitors in tendon-related connective tissue. J Appl Physiol (1985) 96:861–864

Kubo K, Morimoto M, Komuro T, Tsunoda N, Kanehisa H, Fukunaga T (2007) Age-related differences in the properties of the plantar flexor muscles and tendons. Med Sci Sports Exerc 39:541–547

Lee WY, Lui PP, Rui YF (2012) Hypoxia-mediated efficient expansion of human tendon-derived stem cells in vitro. Tissue Eng Part A 18:484–498

Lee CH, Lee FY, Tarafder S, Kao K, Jun Y, Yang G, Mao JJ (2015) Harnessing endogenous stem/progenitor cells for tendon regeneration. J Clin Invest 125:2690–2701

Legerlotz K, Dorn J, Richter J, Rausch M, Leupin O (2014) Age-dependent regulation of tendon crimp structure, cell length and gap width with strain. Acta Biomater 10:4447–4455

Lehner C, Gehwolf R, Ek JC, Korntner S, Bauer H, Bauer HC, Traweger A, Tempfer H (2016) The blood-tendon barrier: identification and characterisation of a novel tissue barrier in tendon blood vessels. Eur Cell Mater 31:296–311

Lejard V, Blais F, Guerquin MJ, Bonnet A, Bonnin MA, Havis E, Malbouyres M, Bidaud CB, Maro G, Gilardi-Hebenstreit P, Rossert J, Ruggiero F, Duprez D (2011) EGR1 and EGR2 involvement in vertebrate tendon differentiation. J Biol Chem 286:5855–5867

Lener T, Gimona M, Aigner L, Borger V, Buzas E, Camussi G, Chaput N, Chatterjee D, Court FA, Del Portillo HA, O'Driscoll L, Fais S, Falcon-Perez JM, Felderhoff-Mueser U, Fraile L, Gho YS, Gorgens A, Gupta RC, Hendrix A, Hermann DM, Hill AF, Hochberg F, Horn PA, de Kleijn D, Kordelas L, Kramer BW, Kramer-Albers EM, Laner-Plamberger S, Laitinen S, Leonardi T, Lorenowicz MJ, Lim SK, Lotvall J, Maguire CA, Marcilla A, Nazarenko I, Ochiya T, Patel T, Pedersen S, Pocsfalvi G, Pluchino S, Quesenberry P, Reischl IG, Rivera FJ, Sanzenbacher R, Schallmoser K, Slaper-Cortenbach I, Strunk D, Tonn T, Vader P, van Balkom BW, Wauben M, Andaloussi SE, Thery C, Rohde E, Giebel B (2015) Applying extracellular vesicles based therapeutics in clinical trials – an ISEV position paper. J Extracell Vesicles 4:30087

Lewis G, Shaw KM (1997) Tensile properties of human tendo Achillis: effect of donor age and strain rate. J Foot Ankle Surg 36:435–445

Lomas AJ, Ryan CN, Sorushanova A, Shologu N, Sideri AI, Tsioli V, Fthenakis GC, Tzora A, Skoufos I, Quinlan LR, O'Laighin G, Mullen AM, Kelly JL, Kearns S, Biggs M, Pandit A, Zeugolis DI (2015) The past, present and future in scaffold-based tendon treatments. Adv Drug Deliv Rev 84:257–277

Longo UG, Ronga M, Maffulli N (2009) Achilles tendinopathy. Sports Med Arthrosc 17:112–126

Lui PP (2013) Identity of tendon stem cells – how much do we know? J Cell Mol Med 17:55–64

Lui PP (2015) Stem cell technology for tendon regeneration: current status, challenges, and future research directions. Stem Cells Cloning 8:163–174

Lui PP, Chan KM (2011) Tendon-derived stem cells (TDSCs): from basic science to potential roles in tendon pathology and tissue engineering applications. Stem Cell Rev 7:883–897

Maeda T, Sakabe T, Sunaga A, Sakai K, Rivera AL, Keene DR, Sasaki T, Stavnezer E, Iannotti J, Schweitzer R, Ilic D, Baskaran H, Sakai T (2011) Conversion of mechanical force into TGF-beta-mediated biochemical signals. Curr Biol 21:933–941

Maffulli N, Khan KM, Puddu G (1998) Overuse tendon conditions: time to change a confusing terminology. Arthroscopy 14:840–843

Maffulli N, Wong J, Almekinders LC (2003) Types and epidemiology of tendinopathy. Clin Sports Med 22:675–692

Mazzocca AD, McCarthy MB, Chowaniec D, Cote MP, Judson CH, Apostolakos J, Solovyova O, Beitzel K, Arciero RA (2011) Bone marrow-derived mesenchymal stem cells obtained during arthroscopic rotator cuff repair surgery show potential for tendon cell differentiation after treatment with insulin. Arthroscopy 27:1459–1471

McNeilly CM, Banes AJ, Benjamin M, Ralphs JR (1996) Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions. J Anat 189(Pt 3):593–600
Meller R, Schiborra F, Brandes G, Knobloch K, Tschernig T, Hankemeier S, Haasper C, Schmiedl A, Jagodzinski M, Krettek C, Willbold E (2009) Postnatal maturation of tendon, cruciate ligament, meniscus and articular cartilage: a histological study in sheep. Ann Anat 191:575–585
Mendias CL, Gumucio JP, Bakhurin KI, Lynch EB, Brooks SV (2012) Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. J Orthop Res 30:606–612
Mienaltowski MJ, Adams SM, Birk DE (2013) Regional differences in stem cell/progenitor cell populations from the mouse achilles tendon. Tissue Eng Part A 19:199–210
Morita W, Snelling SJ, Dakin SG, Carr AJ (2016) Profibrotic mediators in tendon disease: a systematic review. Arthritis Res Ther 18:269
Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, Schweitzer R (2007) Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development 134:2697–2708
Ni M, Rui YF, Tan Q, Liu Y, Xu LL, Chan KM, Wang Y, Li G (2013) Engineered scaffold-free tendon tissue produced by tendon-derived stem cells. Biomaterials 34:2024–2037
Nourissat G, Berenbaum F, Duprez D (2015) Tendon injury: from biology to tendon repair. Nat Rev Rheumatol 11:223–233
Oliva F, Via AG, Maffulli N (2011) Calcific tendinopathy of the rotator cuff tendons. Sports Med Arthrosc 19:237–243
Oliva F, Via AG, Maffulli N (2012) Physiopathology of intratendinous calcific deposition. BMC Med 10:95
Onambele GL, Narici MV, Maganaris CN (2006) Calf muscle-tendon properties and postural balance in old age. J Appl Physiol (1985) 100:2048–2056
Oryan A, Shoushtari AH (2008) Histology and ultrastructure of the developing superficial digital flexor tendon in rabbits. Anat Histol Embryol 37:134–140
Pajala A, Melkko J, Leppilahti J, Ohtonen P, Soini Y, Risteli J (2009) Tenascin-C and type I and III collagen expression in total Achilles tendon rupture. An immunohistochemical study. Histol Histopathol 24:1207–1211
Park A, Hogan MV, Kesturu GS, James R, Balian G, Chhabra AB (2010) Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers. Tissue Eng Part A 16:2941–2951
Peacock EE Jr (1959) A study of the circulation in normal tendons and healing grafts. Ann Surg 149:415–428
Pingel J, Lu Y, Starborg T, Fredberg U, Langberg H, Nedergaard A, Weis M, Eyre D, Kjaer M, Kadler KE (2014) 3-D ultrastructure and collagen composition of healthy and overloaded human tendon: evidence of tenocyte and matrix buckling. J Anat 224:548–555
Popov C, Burggraf M, Kreja L, Ignatius A, Schieker M, Docheva D (2015a) Mechanical stimulation of human tendon stem/progenitor cells results in upregulation of matrix proteins, integrins and MMPs, and activation of p38 and ERK1/2 kinases. BMC Mol Biol 16:6
Popov C, Kohler J, Docheva D (2015b) Activation of EphA4 and EphB2 reverse signaling restores the age-associated reduction of self-renewal, migration, and actin turnover in human tendon stem/progenitor cells. Front Aging Neurosci 7:246
Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ (2006) Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20:1487–1495
Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE, Hazleman BL (1994) Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis. Ann Rheum Dis 53:367–376
Riley GP, Curry V, DeGroot J, van El B, Verzijl N, Hazleman BL, Bank RA (2002) Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. Matrix Biol 21:185–195

Robinson PS, Huang TF, Kazam E, Iozzo RV, Birk DE, Soslowsky LJ (2005) Influence of decorin and biglycan on mechanical properties of multiple tendons in knockout mice. J Biomech Eng 127:181–185

Rolf CG, Fu BS, Pau A, Wang W, Chan B (2001) Increased cell proliferation and associated expression of PDGFRbeta causing hypercellularity in patellar tendinosis. Rheumatology (Oxford) 40:256–261

Rui YF, Lui PP, Li G, Fu SC, Lee YW, Chan KM (2010) Isolation and characterization of multipotent rat tendon-derived stem cells. Tissue Eng Part A 16:1549–1558

Rui YF, Lui PP, Chan LS, Chan KM, Fu SC, Li G (2011) Does erroneous differentiation of tendon-derived stem cells contribute to the pathogenesis of calcifying tendinopathy? Chin Med J (Engl) 124:606–610

Rui YF, Lui PP, Wong YM, Tan Q, Chan KM (2013a) Altered fate of tendon-derived stem cells isolated from a failed tendon-healing animal model of tendinopathy. Stem Cells Dev 22:1076–1085

Rui YF, Lui PP, Wong YM, Tan Q, Chan KM (2013b) BMP-2 stimulated non-tenogenic differentiation and promoted proteoglycan deposition of tendon-derived stem cells (TDSCs) in vitro. J Orthop Res 31:746–753

Runesson E, Ackermann P, Karlsson J, Eriksson BI (2015) Nucleostemin- and Oct 3/4-positive stem/progenitor cells exhibit disparate anatomical and temporal expression during rat Achilles tendon healing. BMC Musculoskelet Disord 16:212

Salingcarnboriboon R, Yoshitake H, Tsuji K, Obinata M, Amagasa T, Nifuji A, Noda M (2003) Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Exp Cell Res 287:289–300

Sassoon AA, Ozasa Y, Chikenji T, Sun YL, Larson DR, Maas ML, Zhao C, Jen J, Amadio PC (2012) Skeletal muscle and bone marrow derived stromal cells: a comparison of tenocyte differentiation capabilities. J Orthop Res 30:1710–1718

Schiele NR, Marturano JE, Kuo CK (2013) Mechanical factors in embryonic tendon development: potential cues for stem cell tenogenesis. Curr Opin Biotechnol 24:834–840

Schwartz MA (2010) Integrins and extracellular matrix in mechanotransduction. Cold Spring Harb Perspect Biol 2:a005066

Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, Lassar A, Tabin CJ (2001) Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. Development 128:3855–3866

Sharma P, Maffulli N (2006) Biology of tendon injury: healing, modeling and remodeling. J Musculoskelet Neuronal Interact 6:181–190

Sharma RI, Snedeker JG (2012) Paracrine interactions between mesenchymal stem cells affect substrate driven differentiation toward tendon and bone phenotypes. PLoS One 7:e31504

Smith RK, Zunino L, Webbon PM, Heinegard D (1997) The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load. Matrix Biol 16:255–271

Snedeker JG, Gautieri A (2014) The role of collagen crosslinks in ageing and diabetes – the good, the bad, and the ugly. Muscles Ligaments Tendons J 4:303–308

Speed C (2016) Inflammation in tendon disorders. Adv Exp Med Biol 920:209–220

Spiesz EM, Thorpe CT, Chaudhry S, Riley GP, Birch HL, Clegg PD, Screen HR (2015) Tendon extracellular matrix damage, degradation and inflammation in response to in vitro overload exercise. J Orthop Res 33:889–897

Stanley RL, Fleck RA, Becker DL, Goodship AE, Ralphs JR, Patterson-Kane JC (2007) Gap junction protein expression and cellularity: comparison of immature and adult equine digital tendons. J Anat 211:325–334

Stenroth L, Peltonen J, Cronin NJ, Sipila S, Finni T (2012) Age-related differences in Achilles tendon properties and triceps surae muscle architecture in vivo. J Appl Physiol (1985) 113:1537–1544

Sugg KB, Lubardic J, Gumucio JP, Mendias CL (2014) Changes in macrophage phenotype and induction of epithelial-to-mesenchymal transition genes following acute Achilles tenotomy and repair. J Orthop Res 32:944–951

Sugimoto Y, Takimoto A, Akiyama H, Kist R, Scherer G, Nakamura T, Hiraki Y, Shukunami C (2013) Scx+/Sox9+ progenitors contribute to the establishment of the junction between cartilage and tendon/ligament. Development 140:2280–2288

Tan Q, Lui PP, Lee YW (2013) In vivo identity of tendon stem cells and the roles of stem cells in tendon healing. Stem Cells Dev 22:3128–3140

Tempfer H, Traweger A (2015) Tendon vasculature in health and disease. Front Physiol 6:330

Tempfer H, Wagner A, Gehwolf R, Lehner C, Tauber M, Resch H, Bauer HC (2009) Perivascular cells of the supraspinatus tendon express both tendon- and stem cell-related markers. Histochem Cell Biol 131:733–741

Tetta C, Consiglio AL, Bruno S, Tetta E, Gatti E, Dobreva M, Cremonesi F, Camussi G (2012) The role of microvesicles derived from mesenchymal stem cells in tissue regeneration; a dream for tendon repair? Muscles Ligaments Tendons J 2:212–221

Teunis T, Lubberts B, Reilly BT, Ring D (2014) A systematic review and pooled analysis of the prevalence of rotator cuff disease with increasing age. J Shoulder Elbow Surg 23:1913–1921

Thomopoulos S, Genin GM, Galatz LM (2010) The development and morphogenesis of the tendon-to-bone insertion – what development can teach us about healing. J Musculoskelet Neuronal Interact 10:35–45

Thorpe CT, Streeter I, Pinchbeck GL, Goodship AE, Clegg PD, Birch HL (2010) Aspartic acid racemization and collagen degradation markers reveal an accumulation of damage in tendon collagen that is enhanced with aging. J Biol Chem 285:15674–15681

Thorpe CT, Birch HL, Clegg PD, Screen HR (2013) The role of the non-collagenous matrix in tendon function. Int J Exp Pathol 94:248–259

Tokunaga T, Shukunami C, Okamoto N, Taniwaki T, Oka K, Sakamoto H, Ide J, Mizuta H, Hiraki Y (2015) FGF-2 stimulates the growth of Tenogenic progenitor cells to facilitate the generation of Tenomodulin-positive tenocytes in a rat rotator cuff healing model. Am J Sports Med 43:2411–2422

Tsai WC, Chang HN, Yu TY, Chien CH, Fu LF, Liang FC, Pang JH (2011) Decreased proliferation of aging tenocytes is associated with down-regulation of cellular senescence-inhibited gene and up-regulation of p27. J Orthop Res 29:1598–1603

Tsimbouri PM (2015) Adult stem cell responses to Nanostimuli. J Funct Biomater 6:598–622

Usuelli FG, Grassi M, Maccario C, Vigano M, Lanfranchi L, Alfieri Montasio U, de Girolamo L (2017) Intratendinous adipose-derived stromal vascular fraction (SVF) injection provides a safe, efficacious treatment for Achilles tendinopathy: results of a randomized controlled clinical trial at a 6-month follow-up. Knee Surg Sports Traumatol Arthros. https://doi.org/10.1007/s00167-017-4479-9

Veronesi F, Salamanna F, Tschon M, Maglio M, Nicoli Aldini N, Fini M (2016) Mesenchymal stem cells for tendon healing: what is on the horizon? J Tissue Eng Regen Med 40:555

Violini S, Ramelli P, Pisani LF, Gorni C, Mariani P (2009) Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. BMC Cell Biol 10:29

Vogel KG, Ordog A, Pogany G, Olah J (1993) Proteoglycans in the compressed region of human tibialis posterior tendon and in ligaments. J Orthop Res 11:68–77

Voleti PB, Buckley MR, Soslowsky LJ (2012) Tendon healing: repair and regeneration. Annu Rev Biomed Eng 14:47–71

Vorbruggen G, Jackle H (1997) Epidermal muscle attachment site-specific target gene expression and interference with myotube guidance in response to ectopic stripe expression in the developing drosophila epidermis. Proc Natl Acad Sci U S A 94:8606–8611

Wang JH (2006) Mechanobiology of tendon. J Biomech 39:1563–1582

Wang JH, Guo Q, Li B (2012) Tendon biomechanics and mechanobiology – a minireview of basic concepts and recent advancements. J Hand Ther 25:133–140. quiz 141

Wang A, Breidahl W, Mackie KE, Lin Z, Qin A, Chen J, Zheng MH (2013) Autologous tenocyte injection for the treatment of severe, chronic resistant lateral epicondylitis: a pilot study. Am J Sports Med 41:2925–2932

Wang A, Mackie K, Breidahl W, Wang T, Zheng MH (2015) Evidence for the durability of autologous tenocyte injection for treatment of chronic resistant lateral epicondylitis: mean 4.5-year clinical follow-up. Am J Sports Med 43:1775–1783

Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE (2004) Type V collagen controls the initiation of collagen fibril assembly. J Biol Chem 279:53331–53337

Wu H, Zhao G, Zu H, Wang JH, Wang QM (2015) Aging-related viscoelasticity variation of tendon stem cells (TSCs) characterized by quartz thickness shear mode (TSM) resonators. Sens Actuators (Warrendale) 210:369–380

Xu W, Sun Y, Zhang J, Xu K, Pan L, He L, Song Y, Njunge L, Xu Z, Chiang MY, Sung KL, Chuong CM, Yang L (2015) Perivascular-derived stem cells with neural crest characteristics are involved in tendon repair. Stem Cells Dev 24:857–868

Yang G, Rothrauff BB, Tuan RS (2013) Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm. Birth Defects Res C Embryo Today 99:203–222

Yin Z, Chen X, Chen JL, Ouyang HW (2010a) Stem cells for tendon tissue engineering and regeneration. Expert Opin Biol Ther 10:689–700

Yin Z, Chen X, Chen JL, Shen WL, Hieu Nguyen TM, Gao L, Ouyang HW (2010b) The regulation of tendon stem cell differentiation by the alignment of nanofibers. Biomaterials 31:2163–2175

Yu TY, Pang JH, Wu KP, Chen MJ, Chen CH, Tsai WC (2013) Aging is associated with increased activities of matrix metalloproteinase-2 and -9 in tenocytes. BMC Musculoskelet Disord 14:2

Zelzer E, Blitz E, Killian ML, Thomopoulos S (2014) Tendon-to-bone attachment: from development to maturity. Birth Defects Res C Embryo Today 102:101–112

Zhang J, Wang JH (2010a) Characterization of differential properties of rabbit tendon stem cells and tenocytes. BMC Musculoskelet Disord 11:10

Zhang J, Wang JH (2010b) Mechanobiological response of tendon stem cells: implications of tendon homeostasis and pathogenesis of tendinopathy. J Orthop Res 28:639–643

Zhang J, Wang JH (2010c) Production of PGE(2) increases in tendons subjected to repetitive mechanical loading and induces differentiation of tendon stem cells into non-tenocytes. J Orthop Res 28:198–203

Zhang J, Wang JH (2012) BMP-2 mediates PGE(2) -induced reduction of proliferation and osteogenic differentiation of human tendon stem cells. J Orthop Res 30:47–52

Zhang J, Wang JH (2013a) The effects of mechanical loading on tendons – an in vivo and in vitro model study. PLoS One 8:e71740

Zhang J, Wang JH (2013b) Human tendon stem cells better maintain their stemness in hypoxic culture conditions. PLoS One 8:e61424

Zhang J, Pan T, Liu Y, Wang JH (2010a) Mouse treadmill running enhances tendons by expanding the pool of tendon stem cells (TSCs) and TSC-related cellular production of collagen. J Orthop Res 28:1178–1183

Zhang Y, Wang B, Zhang WJ, Zhou G, Cao Y, Liu W (2010b) Enhanced proliferation capacity of porcine tenocytes in low O2 tension culture. Biotechnol Lett 32:181–187

Zhang K, Asai S, Yu B, Enomoto-Iwamoto M (2015) IL-1beta irreversibly inhibits tenogenic differentiation and alters metabolism in injured tendon-derived progenitor cells in vitro. Biochem Biophys Res Commun 463:667–672

Zhang L, Chen S, Chang P, Bao N, Yang C, Ti Y, Zhou L, Zhao J (2016) Harmful effects of leukocyte-rich platelet-rich plasma on rabbit tendon stem cells in vitro. Am J Sports Med 44:1941–1951

Zhou Z, Akinbiyi T, Xu L, Ramcharan M, Leong DJ, Ros SJ, Colvin AC, Schaffler MB, Majeska RJ, Flatow EL, Sun HB (2010) Tendon-derived stem/progenitor cell aging: defective self-renewal and altered fate. Aging Cell 9:911–915

Part VI

Translational Aspects

Challenges and Status of Adipose Cell Therapies: Translation and Commercialization

Tosan E. Ehanire and Adam J. Katz

Contents

Abstract

- There is an obvious clinical need for novel therapies that offer structural and/or functional recovery of tissues lost to disease or trauma.
- The past decade and a half has ushered in a growing number of scientific investigations of the isolation, characterization, and transplantation of adipose-derived cells.
- There is a mounting body of in vitro and in vivo data that highlight the reparative capacity, as well as several additional benefits of adipose-derived cells (ADCs), including convenient isolation and a broad range of clinical application.
- ADCs appear to release important growth factors for tissue repair, modulate the immune system, home to injured tissues, and have the capacity to differentiate along multiple lineage pathways.
- From a translational perspective, a rapidly expanding number of clinical trials using ADCs are underway around the world.
- Clinical testing has revealed both safety and feasibility in the fields of gastroenterology, neurology, orthopedics, reconstructive surgery, and related clinical disciplines.

T. E. Ehanire · A. J. Katz (✉)
Department of Surgery, Division of Plastic and Reconstructive Surgery, University of Florida, College of Medicine, Gainesville, FL, USA
e-mail: Tosan.Ehanire@surgery.ufl.edu; adam.katz@surgery.ufl.edu

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0_56

- Certain paracrine actions of ADCs have brought up significant concern on how these cells may affect tumor growth.
- Important challenges to the continued progress of ADC translation include the need for a consensus approach to scientific methods regarding accurate delineation of subpopulations, optimum methods of isolation, purification, and culture conditions; better defined mechanisms of action to understand poor reproducibility in outcomes; and recognition of the relatively small sample size of existing clinical data.
- Lack of a single, universal stem cell marker, patient-to-patient variability, and heterogeneity of ADC populations, combined with multiple widely different protocols of cell isolation and expansion, hinder the ability to precisely identify and analyze biological properties of these cells.
- Commercial infrastructure and scalability of cell manufacturing is nearing the tipping point; however, progress in this field must contend not only with deficiencies in knowledge base and standardization of translation processes but also regulatory considerations in ensuring safety and efficacy according to the various jurisdiction entities, as well as unethical practitioners who promote unproven therapies to vulnerable patients at the financial and even health expense of such subjects.

1 Introduction

Adipose tissue as a bio-resource. Previously perceived as dormant, adipose tissue has emerged over the last 16 years as a premier source of cells for evolving tissue engineering and regenerative therapies (Kapur et al. 2015). Because of their origin, adipose-derived cells (ADCs) do not carry the ethical overhead often present in the heated debates involving embryonic stem cells. They are one of several cell types garnered from several differing sources collectively known as adult stem cells. Recently, the idea that these cells are indeed true stem cells has come into question. Although they may not have the developmental flexibility and extended differentiation capacity seen in embryonic stem cells, their human application in the clinic is more likely in the near future. Indeed adipose tissue is now a widely studied tissue in the context of regenerative medicine and has been the subject of preclinical studies and clinical studies directed toward numerous applications (Minteer et al. 2015). A comprehensive review of worldwide registries involving new clinical applications of cell-based treatments reveals an extraordinary increase over the last decade in the number and proportion of such trials that are conducted using cells from adipose tissue (Li et al. 2014).

Since the identification of putative stem cells in the bone marrow over 40 years ago, they have become a standard in the field of tissue engineering and regenerative medicine (Friedenstein et al. 1968; Lindroos et al. 2011; Kokai et al. 2014). Adipose-derived cells (ADCs) are similar to bone marrow-derived stem cells (BMSCs) in that they are capable of maturing toward multiple mesodermal tissue types, including

bone, cartilage, and adipose. They are immunosuppressive, and they show similar surface protein marker expression. ADCs are unique from BMSCs in that they possess the added bonus of ease of harvest and relatively greater yield. ADC harvest takes place through the relatively easy and noninvasive process of liposuction, and cells can be isolated in real time in clinically applicable numbers without the need of expansion in culture.

Adipose tissue: Cell composition, form, and function. Adipose tissue is histologically considered a specialized connective tissue that typically represents 15–20% of total body weight, with this number being notably higher in women (Junqueira and Carneiro 2005). Adipocytes, the terminally differentiated parenchymal cell of adipose tissue, function as a reservoir for water insoluble, calorically dense neutral fats – mainly triglycerides. Triglycerides have twice the caloric density of proteins or carbohydrates and can be concentrated in fat cells without adverse osmotic effects, making adipocytes the most efficient form of nutrient storage. The average human can sustain a 20–40-day fast based on the energy from small amounts of fat. By comparison, energy stored as free glucose can sustain the human body for only a few minutes, and energy stored as glycogen is sufficient to support an overnight fast.

Adipocytes are also a key regulator of the body's overall energy metabolism. The metabolic activity of adipocytes works in concert with both the nervous and endocrine systems to release hormones and various important substances. The physical properties of adipocytes confer various functions. Adipose tissue offers thermal insulation and cushion and keeps organs in place intra-abdominally, shapes the body in the subcutaneous layer, and acts as shock absorbers in the soles and palms. There are well-known two types of adipose tissue: white adipose tissue (WAT) being the more common, composed of cells with one very large droplet of fat, and brown adipose tissue (BAT), composed of cells with multiple lipid droplets and an abundance of mitochondria. Each of these cell types has unique features that confer unique function in the body that are discussed in more detail below. A third lesser known subset of adipose cells, the beige adipocytes (induced BAT or iBAT) that garner features from both WAT and BAT, is also discussed in brief below (Harms and Seale 2013).

White adipose tissue and brown adipose tissue: Form and function. WAT is designed for long-term energy storage. As WAT differentiates from embryonic mesenchymal cells, it develops the appearance of fibroblasts with the accumulation of lipid droplets in their cytoplasm. The mature adipose cells are large, are polyhedral, and each contain a single large lipid droplet for triglyceride storage that nearly encompasses the entire cytoplasmic space, with much smaller lipid droplets visible on TEM. Adipocytes are incorporated in an arrangement of thin external lamina made up of collagen type IV, a feature that is unique among connective tissue cells. Connective tissue constituted of a vascular and nerve network, fibroblasts, macrophages, and other cells divides adipose tissue into incomplete lobules. It forms reticular fibers that fabricate an interwoven network supporting individual fat cells, binding them together. Many organs throughout the body contain and are surrounded by adipose tissue in a pattern of distribution that changes significantly through childhood and adult life with sex hormone determined variability.

Adipocytes are central to the storage and mobilization of lipids. WAT stores triglycerides sourced from dietary fats delivered as chylomicrons, from the products of liver synthesis transported as VLDL, and from local synthesis of free fatty acids and glycerol from glucose that enter the cell by active transport and diffusion. The process of lipid uptake, storage, synthesis, and mobilization is a concerted process influenced by insulin, norepinephrine, glucagon, growth hormone, and various other hormones. The primary target for norepinephrine mediated fatty acid and glycerol mobilization is hormone-sensitive lipase. This enzyme breaks down triglycerides harbored in lipid droplets, after which the free fatty acid diffuses into the capillary system and transports throughout the body bound to carrier protein, albumin. Adipocytes also function as a secretome by being the unique producers of leptin, a hormone that regulates satiety and fat production, as well as numerous other cytokines including pro-inflammatory cytokines. This underlies the mild chronic inflammatory state associated with obesity due to excess formation of adipose tissue.

BAT also originate from embryonic mesenchyme, the same precursor cells that give rise to skeletal muscle cells and a subpopulation of WAT (Harms and Seale 2013). The developmental resemblances between skeletal muscle and BAT are seen in the similar gene expression profile (Myf5 and Pax 7, known to be skeletal myogenic markers) of BAT precursor cells and related mitochondrial proteomes. The name brown adipose fat stems from the appearance garnered by the abundance of cytochrome pigment containing mitochondria among many small lipid droplets in the cytoplasm, as well as the densely packed arrangement of adipocytes around large capillaries present in the tissue. BAT partitions in more defined lobules compared to white adipose tissue, and brown adipocytes are polygonal and relatively small compared to white adipocytes. BAT sparsely distributes in the human adult around the kidneys, adrenal glands, aorta, and mediastinum. It emerges earlier than WAT during fetal development and is at maximal relative body weight at birth, accounting for 2–5% of newborn body weight, distributed in the back, neck, and shoulders. BAT undergoes apoptosis and involution in early life but remains at relatively increased levels and activity in lean adults.

The primary influence over BAT function is the sympathetic nervous system, where norepinephrine stimulates non-shivering thermogenesis to produce heat. Similar to WAT, norepinephrine targets hormone-sensitive lipase to liberate free fatty acid and glycerol from triglycerides stored in lipid droplets. Specialized to brown adipocytes, however, is the immediate metabolism of free fatty acids and the much greater levels of thermogenin or uncoupling protein-1 (UCP1) located in BAT mitochondria inner membrane. This transmembrane protein allows the recycling of protons used in aerobic metabolism, which generates relatively increased oxygen consumption and heat production. Brown adipocytes are therefore uniquely poised to raise temperature within the tissue, warm the locally circulating blood, and distribute heat through the body.

Beige adipocytes (iBAT) are clusters of UCP1-expressing cells that develop within WAT. iBAT are recruited to have thermogenic capacity in response to various stimuli such as beta-adrenergic receptor or peroxisome proliferator activated receptor-gamma agonists. iBAT have features similar to BAT in addition to UCP1

expression including multilocular lipid droplet morphology and high mitochondrial content. Key distinctions are that iBAT and BAT are derived from different embryonic precursors and that iBAT are regulated differentially – by targeted activation – whereas thermogenic capacity of BAT autonomous (Harms and Seale 2013). Other functional distinctions are yet to be further elucidated.

Adipose tissue cell composition: Cell populations and method(s) of isolation. The 1960s heralded new recognition for adipose tissue in science outside of its traditional function of energy storage and production. In 1964, Rodbell et al. described the dissociation and separation of adipose tissue into a single-cell suspension in a rodent (Rodbell 1964). The 1980s and 1990s ushered in further study on the topic of adipose tissue cellular constituents in the context of understanding obesity and fat grafting. Subsequently, the study of its different cellular components has expanded our understanding of the adipocyte precursor cells (i.e., the preadipocyte) and its differentiation program (Loffler and Hauner 1987; Teichert-Kuliszewska et al. 1992; Petruschke and Hauner 1993). The preadipocyte became a focal point for the emerging field of tissue engineering in the late 1990s to early 2000s when the idea that adipose tissue contained progenitor/stem cells with the ability to differentiate into lineages other than adipocytes emerged (Katz et al. 1999; Patrick Jr. et al. 1999; Kimura et al. 2003; von Heimburg et al. 2003; Frye et al. 2005). In 2000, Halvorsen and colleagues (2000) described the osteogenic differentiation of adipose-derived stromal cells, and in 2001, Zuk and colleagues (2001) published findings showing that adipose tissue was a novel source of mesenchymal stem cells with multi-lineage differentiation potential.

The terminology and language surrounding adipose-derived cells remain an area of confusion and non-consensus despite efforts to the contrary (Bourin et al. 2013). For many, the use of the term "stem" cell persists, despite an extensive body of literature that fails to support this label to an extent of scientific rigor similar to the hematopoietic stem cell (Weissman and Shizuru 2008). As the field has evolved, we have gained a better understanding of variables that impact cell identity/subpopulations within adipose-derived cells, and there is no doubt that this trend will continue.

Adipose tissue can be considered to be composed of two main parts: cells and extracellular matrix (ECM). The latter will not be addressed herein. After enzymatic dissociation of adipose tissue, followed by density layering and filtration, the cellular partition of adipose tissue can be further divided into (1) an adipocyte fraction (i.e., mature, lipid-bearing adipocytes) and (2) a "stromal vascular fraction" (SVF), as previously described by Rodbell. Although adipocytes constitute almost 90% of adipose tissue volume, adipose tissue that is dissociated enzymatically yields a heterogeneous population of many other cell types. Assuming removal of erythrocytes, the SVF is composed of leukocytes, stromal cells, and vascular-related cells (Gronthos et al. 2001; Katz et al. 2005; Mitchell et al. 2006). As such, the SVF may actually best be described as a leuko-vascular stromal fraction (LVSF). This distinction is important, as the role of leukocytes (e.g., macrophages) within the LVSF may in fact play a critical role in paracrine signaling processes related to inflammation, angiogenesis, and more (Arnold et al. 1987; Badylak et al. 2008; Corliss et al. 2016).

To omit this from the "standard" terminology in scientific communications is to potentially ignore a critical mechanism of action ascribed to SVF cells.

LVS cells (aka SVF) are freshly isolated cells from adipose tissue that have not been plated into culture or undergone any type of cell separation or isolation methods (other than removal of erythrocytes). These cells are known to be a heterogeneous collection of several different phenotypes based on flow cytometry including B and T lymphocytes, endothelial cells (mature cells and progenitors), fibroblasts, macrophages, pericytes, preadipocytes, and related populations. When LVS cells are placed into culture on standard plastic surfaces, leukocyte and endothelial populations do not attach, and the resulting cell population will reflect a gradual homogeneity with time in culture. Typically, adipose-derived LVS cells that have been placed into culture are referred to as ASCs (adipose stromal cells). However, we now know that the specific type of surface (e.g., plastic, gelatin coated, fibronectin coated, dermal matrix) and/or method (e.g., suspension or surface adherent) used for culture can impact the specific phenotype(s) of resulting cells (Dromard et al. 2011; Nunes et al. 2013). Even more, the difference in the resulting phenotypes may correlate to differences in biological behaviors. As such, it is slightly misleading to refer to all culture-expanded LVS cells as "ADCs" or at least do so without further description of methodology. In any case, it is important for those working in the field to understand that all "ADCs" are not the same, and by describing and discussing them as though they are, we are likely impeding progress of the field. At the very least, it is imperative for each of us to describe the specific methodology involved in the isolation and culture of adipose-derived cells, addressing issues including but not limited to:

- Suspension culture or surface-adherent culture?
  - If surface adherent, what surface?
- Plating density
  - Based on total nucleated cells? Or total viable nucleated cells?
    - How was viability determined and quantified?
- Serum type and percentage
- Additive factors (e.g., growth factors, cytokines)?
- Methods of characterization of resulting cell population
  - For example, immunophenotype by flow
- Which antibodies used?
- Functional assays?

2 Translation

Overview. The number of clinical trials evaluating the safety and efficacy of ADCs in tissue repair and regeneration increases significantly each year, spanning a broad range of applications. There are well over 200 studies underway throughout Asia, Europe, and North and South America, each operating under different regulatory bodies. Study sponsors include academic hospitals, governmental funding agencies,

and private biotechnology corporations. These investigations are based on the demonstrated immunomodulatory, angiogenic, and differentiative properties of ADCs in addressing a diverse selection of conditions including soft tissue defects, autoimmune disease, conditions associated with endocrine dysfunction, gastrointestinal pathology, cardiovascular diseases, and neurologic disorders (Bajek et al. 2016). The study of ADCs has been pursued using a wide variety of methods. Allogenic and autologous ADCs, SVF, the use of additive treatments (bone marrow, growth factor, fibrin glue), variable doses, variable methods of harvest, systemically and locally administered formulations, and variable degrees of cellular manipulation prior to patient introduction have all been described. Overall, there have been no reported major adverse effects (Gir et al. 2012). Key findings from ADC translational research are presented in this section, grouped by mechanism of action.

Tissue repair and regeneration: Arthritis, bone regeneration, chronic wounds, cartilage regeneration, and cosmetic reconstruction. Soft tissue repair is a logical application, and theoretically the simplest application, for adipose-derived cell therapies since the isolated cells presumably do not need to display trans-differentiation potential (Gimble et al. 2010). Treatment of chronic or otherwise difficult-to-heal wounds is being aggressively pursued. Preclinical in vivo wound healing studies in rodent models revealed accelerated wound repair, increased angiogenesis, reduced fibrosis, and improved collagen organization all attributed to ADC paracrine effects by secreting angiogenic cytokines, growth factors, and matrix metalloproteinases (Gir et al. 2012). These data fueled many trials in the clinical setting. Both SVF and ADCs have been approved and employed in clinical trials involving musculoskeletal degeneration, lipodystrophy, Perry-Romberg disease, and cosmetic reconstruction. Overall, clinical studies remain limited by a relatively low number of patients and a reliance on historical case controls as opposed to randomization protocols.

Anecdotal and clinical reports have described treatment of chronic fibrotic postradiation wounds with autologous human SVF cells (Rigotti et al. 2007) in combination with angiogenic growth factor (FGF2) and artificial dermis (Akita et al. 2010a, b). Functionally significant postradiation necrosis in both bone and soft tissue exhibited symptomatic and cosmetic improvement after treatment with fat graft including rich lipoaspirate with demonstrated presence of ADCs. Improved wound healing was observed; wounds were more supple, suffered less contraction, and were characterized by increased microcapillary growth on histology. Adipocyte-differentiated autologous ADCs have proven to be safe and efficacious when implanted into depressed scars in a safety trial evaluating outcomes in treating cosmetic defects (Kim et al. 2011).

Preclinical data suggest that both SVF and ASC have the ability to promote repair in skeletal tissues. Studies in rodent models demonstrate ASC differentiation into osteoblast after implantation with BMP2 and scaffolds into critical-sized calvarial defects. Systemic injection of ADCs into rodent models of bone loss reduced bone resorption by increasing the numbers of osteoblasts and osteoclasts. These findings have been extended into clinical practice (Gimble et al. 2012). There are several reports supporting the use of SVF and ADCs in reconstructing bony defects. These

treatments typically involve the use of SVF and/or ASC as a component of some combination of bone auto- or allograft or titanium cage, with seeded bio-resorbable scaffold material and/or bone morphogenetic protein-2. Case reports have shown injecting ADCs mixed with platelet-rich plasma, hyaluronic acid, and calcium chloride directly into the hip and knee of patients with avascular necrosis of the femoral head, and meniscus cartilage degeneration confers visible filling of bony and cartilaginous defects on MRI with symptomatic improvement in mobility (Pak et al. 2016). Calvarial defects were studied by Thesleff et al. who performed cranioplasty with autologous ADC seeded in beta-tricalcium phosphate for four patients and demonstrated bone regeneration on computed tomography scan (Thesleff et al. 2011). Other separate reports have shown success with similar formulations of ASC in reconstructing bone including maxilla for dental implantation (Prins et al. 2016) and bi-orbito-zygomatic hypoplasia in Treacher Collins. Saxer et al. demonstrated the successful use of SVF loaded onto ceramic granules within fibrin gel to treat humeral fractures where 1-year biopsy revealed osteogenically reconstructed bone at the fracture site (Saxer et al. 2016). These reports indicate potential for SVF/ASC in eliminating the need for large structural allograft and high donor site morbidity that accompanies standard construct of osteocutaneous free flaps (Gir et al. 2012). While these studies support the promise of human ADCs for hard tissue regeneration, preclinical mechanistic studies and randomized controlled clinical trials merit evaluation in the future.

Case reports and several published clinical trials have reported favorable outcomes with local treatment of viscera-cutaneous fistulas using ADCs. Expanded ADCs have been used in fistula repair from Crohn's and other gastrointestinal fistula etiologies. The patients have been shown to benefit from cells isolated mixed with fibrin glue injected into the fistulous tract. Clinical trials demonstrated no adverse effects, and up to 75% of ASC-treated subjects achieved complete reepithelialization of the external opening (Garcia-Olmo et al. 2003, 2005, 2009, 2010; Garcia-Arranz et al. 2016). These studies were unable to determine a correlation between dosing of ASC and clinical effect. A similar formulation of ADCs has also been shown to successfully treat tracheomediastinal fistula as a complication of lung cancer (Alvarez et al. 2008).

Clinical trials evaluating ADCs and fat grafting for cosmetic applications, namely, breast reconstruction or augmentation, have enrolled the greatest number of patients. Fat grafting, although a commonly performed procedure with an extensive number of clinical applications, often faces limitations such as variable success with volume retention, fibrosis, and necrosis. ADC incorporation in lipo-injection in a technology named cell-assisted lipotransfer (CAL) has been an appealing addition to the traditional procedure with hopes of addressing these issues. SVF fat grafting specifically offers potential for concomitant isolation and use in a point-of-care delivery system. While some studies have revealed better retention of volume at up to 1 year after surgery, and relatively higher patient and surgeon satisfaction (Matsumoto et al. 2006; Yoshimura et al. 2008, 2010), others demonstrated no significant improvement compared to unenhanced fat grafting (Peltoniemi et al. 2013). Similar conflicting results were demonstrated in facial lipofilling for various

indications including lipoatrophy, Parry-Romberg syndrome, and craniofacial macrosomia. Variable results in volume retention, patient satisfaction, symmetry, scarring, deformity, and skin improvements were identified after fat grafting with ADC (Sterodimas et al. 2009; Mailey et al. 2013; Tanikawa et al. 2013). It is theorized that SVF cells, by releasing angiogenic cytokines and providing a scaffold for additional cells to organize and differentiate, improve the autologous fat graft viability and implantation. These properties are believed to benefit outcomes in fat grafting by improving vascularity of implanted fat graft, producing scavenge reactive oxygen species, and enhancing adipocyte progenitor survival, proliferation, and differentiation. More work is needed to further validate these clinical findings with control groups and longer follow-ups in order to demonstrate statistically significant results.

Autoimmune. In a manner similar to BMSCs, ADCs are known to modify their environment by exerting significant immunomodulatory effects. In vitro studies have demonstrated both suppression of T-cell proliferation and increased proliferation of T-regulatory cells by ADCs. Independent studies have produced evidence that ADCs produce differential cytokine-mediated feedback loops where a pro-inflammatory response is suppressed in mononuclear cells but stimulated in purified T cells. Studies have also suggested variable immunomodulatory effects depending on the differentiation status of the ASC. Reportedly, undifferentiated ADCs are immunopriviledged and do not display HLA-DR, whereas in a differentiated state, this surface antigen is upregulated along with notable increase in IFN-gamma secretion. Variable immunomodulatory function has also been illustrated with variable culture milieu where lymphoid proliferative response to ADCs changed in the presence of cardiomyocytes (Gimble et al. 2012).

In vivo studies have evaluated the immunomodulatory effects of ADCs in autoimmune and immunological diseases including mouse models of systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (Gimble et al. 2010). Given findings demonstrating significantly decreased mortality in vivo with murine models of GVDH with hematopoietic stem cell and ASC co-transplantation (Yanez et al. 2006), ADCs have been investigated in several clinical studies on GVHD. GVHD is an iatrogenic induced immune disorder that causes considerable morbidity to recipient due to donor lymphocyte destruction of host cells following bone marrow or hematopoietic stem cell transplantation. The potential role of culture-expanded ADCs in prolongation of transplant-related immunosuppressive therapy and suppression of steroid-resistant GVHD has been demonstrated (Fang et al. 2007a, b). Infusion of ADCs improved disease complications in four of six patients studied for a median of 40 months post-procedure. Similar results have been demonstrated in refractory cases of pure red cell aplasia after incompatible hematopoietic stem cell transplantation and chronic autoimmune thrombocytopenic purpura (Fang et al. 2009a, b). Patients achieved remission, and no adverse effects were observed. ADCs have also been demonstrated to improve neurologic function in patients with multiple sclerosis. A series of case studies using intravenous infusion of autologous SVF reported improved cognition and an almost complete reduction of extreme spasticity (Riordan et al. 2009). Rheumatoid arthritis treatments were

examined in a case report where a patient as treated with intravenous infusion of autologous stromal vascular fraction cells isolated from liposuction procedure. The patient reported considerable resolution of joint pain and stiffness, decreased rheumatoid factor. The use of ADCs to treat autoimmune conditions is a promising prospect; however, the mechanism underlying ADC immunomodulatory function remains an area of active investigation (Gimble et al. 2012). Additional safety and efficacy studies are needed to set the stage for large-scale, randomized controlled clinical trials (Kebriaei and Robinson 2011).

Endocrine: Diabetes mellitus and complications. Insulin-dependent DM has attracted substantial attention as an autoimmune disease amenable to ADC intervention via the mechanism of regenerating endogenous insulin production. Human ADCs isolated from the eyelid and analyzed for their neural crest-like origins were found capable of induced differentiation into insulin-expressing cells that improved glucose sensitivity in a mouse model. Other in vitro data revealed improved engraftment of pancreatic beta-islets in diabetic mice when co-implanted with ADCs. Grafts had enhanced vascularization and reduced immune cell infiltration (Gimble et al. 2012). These data spurred the study of ADCs in treating insulinopenic patients. Allogenic ADCs have been demonstrated to treat DM type 1 in two small sample (5–10 patient) studies (Gir et al. 2012). Patients treated with allogenic cells cultured and differentiated into insulin-producing mesenchymal cells transfused mixed with unfractionated autologous cultures of bone marrow. No adverse effects were reported; all subjects were reported to be healthier and gaining weight and had a gradual decrease in insulin requirement, a decrease in HbA1c by an average of 1%, and increased production of endogenous insulin. ADCs, with easy and repeatable access for isolation, have clear advantage over BMSC for improved DM treatment options; however, confirmatory studies and randomized controlled clinical trials will be required.

Cardiovascular: Ischemic limb and myocardial infarction. ADCs support of angiogenesis and their differentiation potential toward endothelial cells along with participation in blood vessel formation is confirmed in the literature. Formation of functional vascularization by these cells was confirmed with in vivo models of myocardial infarction and regeneration of endothelium. There has been increasing attention paid to the application of SVF/ADCs for the treatment of ischemic injuries, with a particular interest in myocardial infarction. Rodent studies where SVF or ADCs were injected systemically or directly into the myocardium have demonstrated improved ejection fraction, wall thickness, and improved cardiac recovery following infarcts. On tissue analysis, there was reduced infarct size and increased capillary density. While the work with animal models is promising, the modest overall degree of improvement in these myocardial ischemia models has potentially deterred more aggressive clinical translation protocols. Further preclinical studies documenting the paracrine and/or differentiation mechanism of ADC and SVF cell cardiac repair and their efficacy will likely accelerate the clinical translation process. Similar stagnation is seen in the use of ADCs for limb ischemia and stroke models. Preclinical data indicated improved hind limb recovery after ischemia in murine models. Mechanism of action is posited to be due to paracrine effects of the cytokine

profile released by ADCs induced in a hypoxic environment which promotes cell proliferation and vasculogenesis (Gimble et al. 2012; Bajek et al. 2016).

Neurologic disorder: Central nervous system, multiple sclerosis, Parkinson's, spinal cord ischemia, and stroke. In vitro data suggests that ADCs promote the regeneration of central nervous system cells and show neuroprotective activity by secreting brain-derived neurotrophic factor, glial-derived neurotrophic factor, nerve growth factor, and IGF. The translational research on ADC participation in the formation of functional neurons is contradictory. Rodent models of cerebral ischemia showed their potential for therapeutic application when ADCs were induced to undergo neuronal differentiation and resulted in improved neurological recovery and reduced infarct size relative to controls. In clinical data, however, efficacy of ADC in nerve regeneration could not be determined. ADCs were studied in a case report of patients with spinal cord injury who were treated with intravenous infusion of autologous adipose-derived stem cells (Gir et al. 2012). No adverse effects were observed, but only half the patient had improved motor function. More research with the use of ADCs in nerve injuries is needed in order to confirm their participation in neuronal regeneration (Bajek et al. 2016).

Strategies and infrastructure for translation of adipose-derived cell therapies. The scientific merits, mechanisms, and differences related to the use of SVF cells versus culture-expanded ADCs remain to be clarified. Nevertheless, the two cell types (i.e., SVF, ADCs) are associated with different translational mechanisms as it pertains to infrastructure, cost, and perhaps regulatory hurdles. Recently, there has been an emerging focus on the biology and clinical translation of LVS cells. The use of autologous freshly isolated, uncultured LVS cells at the "point-of-care" in real time during a "same surgical procedure" has important advantages with respect to the use of culture-expanded cells: less chance for contamination, more rapid processing and clinical application, and less associated costs. Some disadvantages that may be associated with this approach are insufficient cell dose if high cell numbers are needed, challenges with cell "product" variability and inconsistent results, and/or lack of therapeutic efficacy. At present, there are few, if any, studies in the literature directly comparing the efficacy/potency of LVS cells versus ADCs. Furthermore, it is also important to emphasize that many published reports inaccurately use the terms ADCs or adipose stem cells to refer to SVF/LVS.

Certain biological differences are now readily apparent between LVS cells and ADCs. For example, since LVS cells contain endothelial cells and endothelial progenitors, they demonstrate more effective and efficient vasculogenesis compared to culture-expanded cells that quickly lose endothelial phenotypes when adhered to plastic. In addition, the leukocyte fraction of LVS cells, which is not present in culture-expanded cells, may confer anti-inflammatory benefits for therapeutic objectives that culture-expanded cells do not. For example, Semon et al. reported that an intraperitoneal dose of one million SVF/LVS cells was more effective than ADCs in inhibiting experimental autoimmune encephalomyelitis progression (Semon et al. 2014). Autologous SVF/LVS cells also had a more evident positive effect (improvement of range of motion and pain for all patients) than allogeneic ADCs in a model of hip dysplasia in dogs (Marx et al. 2014). In yet another study, Jurgens et al. (2013)

demonstrated best performance of freshly isolated SVF/LVS cells in comparison with cultured ADCs, in promoting cartilage and subchondral bone regeneration in a goat model. All of these studies have shown safety and feasibility of point-of-care SVF/LVS cell therapies with no adverse effects related to the treatments reported.

In summary, it remains to be determined whether putative functional differences between LVS cells and ADCs have relevance for specific clinical applications. Additional preclinical data and clinical trials comparing both cell types may help to clarify these questions.

Researchers and biotech companies have explored various methods and devices for point-of-care isolation of LVS cells. In general, LVS cells are isolated from adipose tissue harvested with lipoplasty and processed in devices (ranging from automated to manual) that enzymatically and/or mechanically dissociate the tissue to varying degrees in a closed system, collecting stromal cells (as single-cell suspensions (enzymatic) or as micro-"clumps" (mechanical) for subsequent use. In general, LVS cells can be safely isolated, quantified, and characterized at the point of care in approximately 70–90 min, enabling isolation and treatment to occur in the same procedure. Although direct comparisons between different devices are scarce, at least one study demonstrates a range of cell yields of 500,000–1,000,000 cells per gram of lipoaspirate tissue with >80% viability (Aronowitz et al. 2015). At least three different companies (Cytori Therapeutics, The GID Group, and Tissue Genesis) have devices in late-stage FDA testing for various clinical indications, but at present, none are formally approved for use in the USA (see www.clinicaltrials.gov).

3 Commercialization Risk

Federal regulation of cell therapies. Regulations that govern ADC translation and commercialization vary according to jurisdiction. The USA, EU, and Japan are the three largest and most rigorously regulated pharmaceutical markets internationally. The Center for Biologics and Evaluation and Research (CBER) section of the US FDA oversees stromal and stem cell products in the USA, the European Medicines Agency (EMA) acts as the centralized regulatory agency for member states of the EU, and the Pharmaceutical and Medical Devices Agency serves as the equivalent governing body in Japan (Gimble et al. 2012). These and other national regulatory authorities have developed guidelines for industry specifically for adult cell production that are generally considered biological products as opposed to devices or drugs. These guidance documents continue to be revised and updated as new evidence and products are evaluated.

In the USA specifically, there has been much debate and controversy related to the regulation of cell therapies – and autologous cell therapies in particular. At the core of the controversy is whether it is the government's purview to regulate how an individual's own cells and tissues are used for that same individual and, if so, to what extent. Strong feelings and arguments have been presented on both "sides" of this issue, most recently at a 2-day public hearing help by the FDA in September, 2016. Key issues that await further clarification relate to regulatory issues of "minimal

manipulation," "homologous use," and the concept of "same surgical procedure." At present, the FDA has released several draft guidance documents pertinent to the use of adipose-derived cell therapies that reflect their current thinking on these matters:

- http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm427795.htm
- http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm427692.htm
- http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm419911.htm
- http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM469751.pdf

4 Conclusions

As the regulatory issues regarding cell therapies await further clarification, various clinics around the USA (and world) have proceeded to market "stem cell" and related therapies to patients. Of concern to many is the possibility that some of these clinics may be offering unproven therapies (either safety and/or efficacy) to vulnerable patients, at the patient's own financial (and possibly) health expense (Turner and Knoepfler 2016). Recent reports of severe morbidity and even mortalities related to cell therapies should at the very least make all in the field pause to reflect on what is best for our patients (Ledford 2011, 2016). At the same time, it is critical that the process of innovation and the development of new, improved therapies not be unduly delayed or suppressed. The manner and extent to which these two important concepts will be balanced as the field of cell therapies moves forward remains to be seen, but most certainly represents a challenge of equal importance to further understanding of if and how these cell therapies definitively help to improve peoples' lives.

References

Akita S, Akino K, Hirano A, Ohtsuru A, Yamashita S (2010a) Mesenchymal stem cell therapy for cutaneous radiation syndrome. Health Phys 98(6):858–862

Akita S, Akino K, Hirano A, Ohtsuru A, Yamashita S (2010b) Noncultured autologous adipose-derived stem cells therapy for chronic radiation injury. Stem Cells Int 2010:532704

Alvarez PD, Garcia-Arranz M, Georgiev-Hristov T, Garcia-Olmo D (2008) A new bronchoscopic treatment of tracheomediastinal fistula using autologous adipose-derived stem cells. Thorax 63 (4):374–376

Arnold F, West D, Kumar S (1987) Wound healing: the effect of macrophage and tumour derived angiogenesis factors on skin graft vascularization. Br J Exp Pathol 68(4):569–574

Aronowitz JA, Lockhart RA, Hakakian CS (2015) Mechanical versus enzymatic isolation of stromal vascular fraction cells from adipose tissue. Springerplus 4:713

Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM (2008) Macrophage phenotype as a determinant of biologic scaffold remodeling. Tissue Eng A 14(11):1835–1842

Bajek A, Gurtowska N, Olkowska J, Kazmierski L, Maj M, Drewa T (2016) Adipose-derived stem cells as a tool in cell-based therapies. Arch Immunol Ther Exp 64:443

Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM (2013) Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 15(6):641–648

Corliss BA, Azimi MS, Munson JM, Peirce SM, Murfee WL (2016) Macrophages: an inflammatory link between angiogenesis and lymphangiogenesis. Microcirculation 23(2):95–121

Dromard C, Bourin P, Andre M, De Barros S, Casteilla L, Planat-Benard V (2011) Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres. Exp Cell Res 317 (6):770–780

Fang B, Song Y, Liao L, Zhang Y, Zhao RC (2007a) Favorable response to human adipose tissue-derived mesenchymal stem cells in steroid-refractory acute graft-versus-host disease. Transplant Proc 39(10):3358–3362

Fang B, Song Y, Zhao RC, Han Q, Lin Q (2007b) Using human adipose tissue-derived mesenchymal stem cells as salvage therapy for hepatic graft-versus-host disease resembling acute hepatitis. Transplant Proc 39(5):1710–1713

Fang B, Song Y, Li N, Li J, Han Q, Zhao RC (2009a) Mesenchymal stem cells for the treatment of refractory pure red cell aplasia after major ABO-incompatible hematopoietic stem cell transplantation. Ann Hematol 88(3):261–266

Fang B, Song YP, Li N, Li J, Han Q, Zhao RC (2009b) Resolution of refractory chronic autoimmune thrombocytopenic purpura following mesenchymal stem cell transplantation: a case report. Transplant Proc 41(5):1827–1830

Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP (1968) Heterotopic transplants of bone marrow. Transplantation 6(2):230–247

Frye CA, Wu X, Patrick CW (2005) Microvascular endothelial cells sustain preadipocyte viability under hypoxic conditions. Vitro Cell Dev Biol Anim 41(5–6):160–164

Garcia-Arranz M, Dolores Herreros M, Gonzalez-Gomez C, de la Quintana P, Guadalajara H, Georgiev-Hristov T, Trebol J, Garcia-Olmo D (2016) Treatment of Crohn's-related rectovaginal fistula with allogeneic expanded-adipose derived stem cells: a phase I–IIa clinical trial. Stem Cells Transl Med 5:1441

Garcia-Olmo D, Garcia-Arranz M, Garcia LG, Cuellar ES, Blanco IF, Prianes LA, Montes JA, Pinto FL, Marcos DH, Garcia-Sancho L (2003) Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. Int J Color Dis 18 (5):451–454

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(7):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(1):79–86

Garcia-Olmo D, Herreros D, De-La-Quintana P, Guadalajara H, Trebol J, Georgiev-Hristov T, Garcia-Arranz M (2010) Adipose-derived stem cells in Crohn's rectovaginal fistula. Case Rep Med 2010:961758

Gimble JM, Guilak F, Bunnell BA (2010) Clinical and preclinical translation of cell-based therapies using adipose tissue-derived cells. Stem Cell Res Ther 1(2):19

Gimble JM, Bunnell BA, Guilak F (2012) Human adipose-derived cells: an update on the transition to clinical translation. Regen Med 7(2):225–235

Gir P, Oni G, Brown SA, Mojallal A, Rohrich RJ (2012) Human adipose stem cells: current clinical applications. Plast Reconstr Surg 129(6):1277–1290

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM (2001) Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189(1):54–63

Halvorsen YC, Wilkison WO, Gimble JM (2000) Adipose-derived stromal cells – their utility and potential in bone formation. Int J Obes Relat Metab Disord 24(Suppl 4):S41–S44
Harms M, Seale P (2013) Brown and beige fat: development, function and therapeutic potential. Nat Med 19(10):1252–1263
Junqueira LCUA, Carneiro J (2005) Basic histology: text & atlas. New York, McGraw-Hill Medical Pub. Division
Jurgens WJ, Kroeze RJ, Zandieh-Doulabi B, van Dijk A, Renders GA, Smit TH, van Milligen FJ, Ritt MJ, Helder MN (2013) One-step surgical procedure for the treatment of osteochondral defects with adipose-derived stem cells in a caprine knee defect: a pilot study. Biores Open Access 2(4):315–325
Kapur SK, Dos-Anjos Vilaboa S, Llull R, Katz AJ (2015) Adipose tissue and stem/progenitor cells: discovery and development. Clin Plast Surg 42(2):155–167
Katz AJ, Llull R, Hedrick MH, Futrell JW (1999) Emerging approaches to the tissue engineering of fat. Clin Plast Surg 26(4):587–603. viii
Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC (2005) Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. Stem Cells 23 (3):412–423
Kebriaei P, Robinson S (2011) Treatment of graft-versus-host-disease with mesenchymal stromal cells. Cytotherapy 13(3):262–268
Kim M, Kim I, Lee SK, Bang SI, Lim SY (2011) Clinical trial of autologous differentiated adipocytes from stem cells derived from human adipose tissue. Dermatol Surg 37(6):750–759
Kimura Y, Ozeki M, Inamoto T, Tabata Y (2003) Adipose tissue engineering based on human preadipocytes combined with gelatin microspheres containing basic fibroblast growth factor. Biomaterials 24(14):2513–2521
Kokai LE, Marra K, Rubin JP (2014) Adipose stem cells: biology and clinical applications for tissue repair and regeneration. Transl Res 163(4):399–408
Ledford H (2011) Stem-cell scientists grapple with clinics. Nature 474(7353):550
Ledford H (2016) Boom in unproven cell therapies intensifies regulatory debate. Nature 537 (7619):148
Li MD, Atkins H, Bubela T (2014) The global landscape of stem cell clinical trials. Regen Med 9 (1):27–39
Lindroos B, Suuronen R, Miettinen S (2011) The potential of adipose stem cells in regenerative medicine. Stem Cell Rev and Rep 7(2):269–291
Loffler G, Hauner H (1987) Adipose tissue development: the role of precursor cells and adipogenic factors. Part II: the regulation of the adipogenic conversion by hormones and serum factors. Klin Wochenschr 65(17):812–817
Mailey B, Saba S, Baker J, Tokin C, Hickey S, Wong R, Wallace AM, Cohen SR (2013) A comparison of cell-enriched fat transfer to conventional fat grafting after aesthetic procedures using a patient satisfaction survey. Ann Plast Surg 70(4):410–415
Marx C, Silveira MD, Selbach I, da Silva AS, Braga LM, Camassola M, Nardi NB (2014) Acupoint injection of autologous stromal vascular fraction and allogeneic adipose-derived stem cells to treat hip dysplasia in dogs. Stem Cells Int 2014:391274
Matsumoto D, Sato K, Gonda K, Takaki Y, Shigeura T, Sato T, Aiba-Kojima E, Iizuka F, Inoue K, Suga H, Yoshimura K (2006) Cell-assisted lipotransfer: supportive use of human adipose-derived cells for soft tissue augmentation with lipoinjection. Tissue Eng 12(12):3375–3382
Minteer DM, Marra KG, Rubin JP (2015) Adipose stem cells: biology, safety, regulation, and regenerative potential. Clin Plast Surg 42(2):169–179
Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24(2):376–385
Nunes SS, Maijub JG, Krishnan L, Ramakrishnan VM, Clayton LR, Williams SK, Hoying JB, Boyd NL (2013) Generation of a functional liver tissue mimic using adipose stromal vascular fraction cell-derived vasculatures. Sci Rep 3:2141

Pak J, Lee JH, Park KS, Jeong BC, Lee SH (2016) Regeneration of cartilage in human knee osteoarthritis with autologous adipose tissue-derived stem cells and autologous extracellular matrix. Biores Open Access 5(1):192–200

Patrick CW Jr, Chauvin PB, Hobley J, Reece GP (1999) Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. Tissue Eng 5(2):139–151

Peltoniemi HH, Salmi A, Miettinen S, Mannerstrom B, Saariniemi K, Mikkonen R, Kuokkanen H, Herold C (2013) Stem cell enrichment does not warrant a higher graft survival in lipofilling of the breast: a prospective comparative study. J Plast Reconstr Aesthet Surg 66(11):1494–1503

Petruschke T, Hauner H (1993) Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrinol Metab 76(3):742–747

Prins HJ, Schulten EA, Ten Bruggenkate CM, Klein-Nulend J, Helder MN (2016) Bone regeneration using the freshly isolated autologous stromal vascular fraction of adipose tissue in combination with calcium phosphate ceramics. Stem Cells Transl Med 5(10):1362–1374

Rigotti G, Marchi A, Galie M, Baroni G, Benati D, Krampera M, Pasini A, Sbarbati A (2007) Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. Plast Reconstr Surg 119(5):1409–1422. discussion 1423–1404

Riordan NH, Ichim TE, Min WP, Wang H, Solano F, Lara F, Alfaro M, Rodriguez JP, Harman RJ, Patel AN, Murphy MP, Lee RR, Minev B (2009) Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. J Transl Med 7:29

Rodbell M (1964) Localization of lipoprotein lipase in fat cells of rat adipose tissue. J Biol Chem 239:753–755

Saxer F, Scherberich A, Todorov A, Studer P, Miot S, Schreiner S, Guven S, Tchang LA, Haug M, Heberer M, Schaefer DJ, Rikli D, Martin I, Jakob M (2016) Implantation of stromal vascular fraction progenitors at bone fracture sites: from a rat model to a first-in-man study. Stem Cells 34 (12):2956–2966

Semon JA, Maness C, Zhang X, Sharkey SA, Beuttler MM, Shah FS, Pandey AC, Gimble JM, Zhang S, Scruggs BA, Strong AL, Strong TA, Bunnell BA (2014) Comparison of human adult stem cells from adipose tissue and bone marrow in the treatment of experimental autoimmune encephalomyelitis. Stem Cell Res Ther 5(1):2

Sterodimas A, Huanquipaco JC, de Souza Filho S, Bornia FA, Pitanguy I (2009) Autologous fat transplantation for the treatment of Parry-Romberg syndrome. J Plast Reconstr Aesthet Surg 62 (11):e424–e426

Tanikawa DY, Aguena M, Bueno DF, Passos-Bueno MR, Alonso N (2013) Fat grafts supplemented with adipose-derived stromal cells in the rehabilitation of patients with craniofacial microsomia. Plast Reconstr Surg 132(1):141–152

Teichert-Kuliszewska K, Hamilton BS, Deitel M, Roncari DA (1992) Augmented production of heparin-binding mitogenic proteins by preadipocytes from massively obese persons. J Clin Invest 90(4):1226–1231

Thesleff T, Lehtimaki K, Niskakangas T, Mannerstrom B, Miettinen S, Suuronen R, Ohman J (2011) Cranioplasty with adipose-derived stem cells and biomaterial: a novel method for cranial reconstruction. Neurosurgery 68(6):1535–1540

Turner L, Knoepfler P (2016) Selling stem cells in the USA: assessing the direct-to-consumer industry. Cell Stem Cell 19(2):154–157

von Heimburg D, Kuberka M, Rendchen R, Hemmrich K, Rau G, Pallua N (2003) Preadipocyte-loaded collagen scaffolds with enlarged pore size for improved soft tissue engineering. Int J Artif Organs 26(12):1064–1076

Weissman IL, Shizuru JA (2008) The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. Blood 112(9):3543–3553

Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M, Bueren JA (2006) Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells 24(11):2582–2591

Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harii K (2008) Cell-assisted lipotransfer for cosmetic breast augmentation: supportive use of adipose-derived stem/stromal cells. Aesthet Plast Surg 32(1):48–55. discussion 56–47

Yoshimura K, Asano Y, Aoi N, Kurita M, Oshima Y, Sato K, Inoue K, Suga H, Eto H, Kato H, Harii K (2010) Progenitor-enriched adipose tissue transplantation as rescue for breast implant complications. Breast J 16(2):169–175

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228

Index

J. M. Gimble et al. (eds.), *Cell Engineering and Regeneration*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-319-08831-0