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Khawaja Husnain Haider
Editor

Handbook of Stem Cell Therapy

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With 119 Figures and 71 Tables

Springer

Editor
Khawaja Husnain Haider
Department of Basic Sciences
Sulaiman AlRajhi University
Al Bukairiyah, Saudi Arabia

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To
My Parents, Brothers, Sisters, Brothers-in-Law, and
my wife DIYA who mothered our two sons:
Mowahid who is the love of my life
and
Anas "the angel of paradise" whose departure from my life is a constant source of inspiration for me to do science.

Foreword

Debilitating and fatal, hereditary degenerative diseases deprive their sufferers of a normal quality of life and life span. An effective treatment must not only repair degenerating cells but replenish degenerated cells with live ones. From the first success of mesenchyme transplantation in dystrophic mice reported in *Muscle & Nerve* 1982; 5: 619–627 unto the current >20 chapters documenting biomedical and clinical advances of mesenchymal stem cell transplantation, it becomes apparent that cell therapy has come of age! Better still, Food and Drug Agency (FDA), European Medicines Agency (EMA), and National Medicine Products Administration (NMPA) have granted several biologic license approvals since July 14, 1990, when the world's first human gene therapy and somatic cell therapy was published in *Lancet* 336:114–115.

Cell therapy is a mainstream regenerative medicine characterized with transplantation of biologics including live cells, genes, factors, and/or a combination of them. It replenishes live cells to degenerative organs, and in allografts, offers the normal genome for genetic complementation treatment. It regenerates tissues and organs. Exosomes secreted from cultured cells are often formulated to become molecular medicine. Since a foreign gene and its derivatives always exert its effect on the cell, cell therapy is the common pathway to good health. Debilitating and fatal diseases with no known cure are often results of polygenic aberration, and only cell therapy rather than individual molecular medicine can be effective.

Cell therapists harvesting the innovations and discoveries of developmental cell biologists since the mid-1950s should not forget their teachings. Somatic cell therapies and stem cell therapies utilize different compositions and methods of treatment. It is through continual research in cell identification, quantity, purity, viability, and potency, especially in cell differentiation and transcription, that stem cell therapies will one day overcome the inadequacy of uncontrolled differentiation and carcinogenicity.

This is the seventh book Professor Haider has edited on key issues of stem cells and stem cell therapies. Professor Haider has devoted more than two decades in these arenas, publishing cutting-edge research with complete dedication and passion. In this book, he has compiled the latest advances of encompassing cell therapies from world experts toward treating cancer, heart failure, Type-II diabetes, aging, muscular dystrophies, wounds, ocular diseases, COVID-19, inflammatory bowel disease, cartilage damage, spinal cord injury, cognitive deficits, and ischemic stroke. This

book is classified as a Major Reference Work consisting of 50 chapters from nearly 45 labs around the globe, from the USA to Canada, Europe, China, Japan, Malaysia, and many other countries. With these elite Editor, Publisher, and Authors working in harmony, the world of science and medicine will look forward to new editions of this landmark undertaking in the years to come.

All these drops into the bucket, some representing the life-long effort of the pioneers, others representing novel discoveries and innovations, will all be collected monumentally; for biologics are evolutionary medicine shared by all animals in the last 500 million years, only to be discovered, isolated, manipulated with human innovation, and formulated as Genetic Cell Therapies to provide mankind with long-term efficacy and lesser side effects than herbs, chemicals, surgery, or radiation.

Cell Therapy Institute
Wuhan, CHINA
August 2022

Peter K. Law, Ph.D., Professor, Founder & Chairman

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About the Editor

Dr. Khawaja Husnain Haider is currently a Professor of Cellular and Molecular Pharmacology (Stem cells and Gene Therapy) and Chairman of the Basic Sciences Department (Medical Program) at Sulaiman AlRajhi University. He has also served as Principal Investigator (PI) and Co-PI on various NIH-funded stem cells research projects. He has been on the editorial boards of various research journals and has served as an invited reviewer for several respected international journals. His research focuses on the use of DNA, miRNAs, and stem cells as "drugs," a topic that has gained popularity in regenerative medicine. He has published more than 300 book chapters, abstracts, and research papers published in various books and leading research journals, including *Circulation*, *Circulation Research*, *Cardiovascular Research*, *Journal Cellular and Molecular Medicine (JCMM)*, the *Journal of Biological Chemistry*, *Cell Cycle*, *Basic Research in Cardiology*, and *Antioxidant Redox Signaling*. He has also given numerous presentations and edited seven books covering various facets of stem cells and their applications from drug to drug development.

Section Editors

Maria Alvarez-Viejo
Unidad de Terapia Celular y Medicina Regenerativa, Servicio de Hematología y Hemoterapia
Hospital Universitario Central de Asturias HUCA
Instituto de Investigación Biosanitaria del Principado de Asturias and Universidad de Oviedo
Asturias, Spain

Beatrice Aramini
Division of Thoracic Surgery, Department of Experimental, Diagnostic and Specialty Medicine–DIMES of the Alma Mater Studiorum University of Bologna, G.B. Morgagni–L. Pierantoni Hospital
Forlì, Italy

Rony Atoui
Division of Cardiac Surgery, Health Sciences North
Northern Ontario School of Medicine
Sudbury, ON, Canada

Oh Young Bang
Department of Neurology
School of Medicine, Sungkyunkwan University
Samsung Medical Center
Seoul, Korea

Khawaja Husnain Haider
Department of Basic Sciences
Sulaiman AlRajhi University
Al Bukairiyah, Saudi Arabia

Mustapha Najimi
Laboratory of Pediatric Hepatology and Cell Therapy
Institut de Recherche Expérimentale et Clinique (IREC)
Université catholique de Louvain (UCLouvain)
Brussels, Belgium

Elio Antonio Prieto González
Centre for Advanced Studies in Humanities and Health Sciences
Interamerican Open University
Buenos Aires, Argentina

ISALUD University, Nutrition Career
Buenos Aires, Argentina

S. Amer Riazuddin
The Wilmer Eye Institute
Johns Hopkins University School of Medicine
Baltimore, MD, USA

Contributors

Marwa M. S. Abbass Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

A. M. Aimaletdinov Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

Büşra Aktaş Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Burdur Mehmet Akif Ersoy University, Burdur, Turkey

Ana Alfonso-Fernández Servicio de Cirugía Ortopédica y Traumatología, Hospital Universitario Marqués de Valdecilla, Santander, Cantabria, Spain

Muhammad Ali The Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abdullah Murhaf Al-Khani Sulaiman AlRajhi Medical School, Al Bukairiyah, Kingdom of Saudi Arabia

Sulaiman Alnasser Pharmacology and Toxicology Department, Unizah College of Pharmacy, Qassim University, Unaizah, Kingdom of Saudi Arabia

Saleh F. Alqifari College of Pharmacy - Department of Pharmacy Practice, University of Tabuk, Tabuk, Saudi Arabia

Mabrouk AL-Rasheedi Al Bukayriah General Hospital, AlQassim, Kingdom of Saudi Arabia

Mateq A. Alreshidi Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukayriyah, Kingdom of Saudi Arabia

Maria Alvarez-Viejo Unidad de Terapia Celular y Medicina Regenerativa, Servicio de Hematología y Hemoterapia, Hospital Universitario Central de Asturias HUCA, Instituto de Investigación Biosanitaria del Principado de Asturias, Universidad de Oviedo, Asturias, Spain

Tiziana Annese Department of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy

Ming Zhang Ao Hua Zhong University of Science and Technology, Wuhan, China

Bruna Araújo Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

Beatrice Aramini Division of Thoracic Surgery, Department of Experimental, Diagnostic and Specialty Medicine–DIMES of the Alma Mater Studiorum, University of Bologna, G.B. Morgagni–L. Pierantoni Hospital, Forlì, Italy

Belma Aslım Department of Biology, Faculty of Science, Gazi University, Ankara, Turkey

Rony Atoui Division of Cardiac Surgery, Health Sciences North, Northern Ontario School of Medicine, Sudbury, ON, Canada

Saeed Azandeh Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran

Vladimir Baklaushev Federal Research Clinical Center of Specialized Medical Care and Medical Technologies of the FMBA of Russia, Moscow, Russia

Federico Banchelli Center of Statistic, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy

Oh Young Bang Department of Neurology, Samsung Medical Center, School of Medicine, Sungkyunkwan University, Seoul, South Korea

S&E Bio, Inc, Seoul, South Korea

Tiffany Barszczewski Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada

Cellular and Regenerative Medicine Centre, BC Children's Hospital Research Institute, Vancouver, BC, Canada

Derek Barthels Department of Pharmaceutical Sciences, Jerry H. Hodge School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, TX, USA

Francico J. Bedoya Department of Molecular Biology and Biochemical Engineering, University of Pablo de Olavide, Sevilla, Spain

Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid, Spain

Mohamed Amine Bekadja Department of Hematology and Stem Cell Therapy, Etablissement Hospitalier Universitaire 1st November, Ahmed Benbella 1 University, Oran, Algeria

Alexander E. Berezin Internal Medicine Department, State Medical University, Zaporozhye, Ukraine

Alexander A. Berezin Internal Medicine Department, Academy of Post-graduating Education, State Medical University, Zaporozhye, Ukraine

Kiran Kumar Bokara CSIR–Centre for Cellular and Molecular Biology, Hyderabad, India

Theo Borgovan Division of Hematology and Oncology, Rhode Island Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, USA

Patrick G. Burgon Department of Chemistry and Earth Sciences, College of Arts and Sciences, Qatar University, Doha, Qatar

Jae Min Cha Department of Mechatronics Engineering, College of Engineering, Incheon National University, Incheon, South Korea

3D Stem Cell Bioengineering Laboratory, Research Institute for Engineering and Technology, Incheon National University, Incheon, South Korea

Weyland Cheng Children's Hospital Affiliated to Zhengzhou University, Henan Children's Hospital, Zhengzhou Children's Hospital, Zhengzhou, China

Rani Chudasama Division of Hematology and Oncology, Rhode Island Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, USA

Roberto D'Amico Center of Statistic, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy

Ekta Dagar CSIR–Centre for Cellular and Molecular Biology, Hyderabad, India

Hiranmoy Das Department of Pharmaceutical Sciences, Jerry H. Hodge School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, TX, USA

Sanat Kumar Dash Department of Mechanical Engineering, Indian Institute of Technology-Madras, Chennai, Tamil Nadu, India

Sofia Domingues Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

Massimo Dominici Division of Oncology, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy

Christof Dörfer Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian-Albrecht's University, Kiel, Germany

Maria Dubrovcakova Biomedical Center, Slovak Academy of Sciences, Bratislava, Slovakia

Sara El Moshy Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Aiah A. El-Rashidy Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Biomaterials Department, Faculty Dentistry, Cairo University, Cairo, Egypt

Azza Ezz El-Arab Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Adegbenro Omotuyi John Fakoya Department of Anatomical Sciences, University of Medicine and Health Sciences, Basseterre, Saint Kitts and Nevis

Karim M. Fawzy El-Sayed Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian Albrechts University, Kiel, Germany

Michael G. Fehlings Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada

Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada

Division of Neurosurgery, Department of Surgery, University of Toronto, Toronto, ON, Canada

Daniel García-Sánchez Grupo de Ingeniería de Tejidos de Cantabria, Dpto. Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IDIVAL, Santander, Cantabria, Spain

Anneh Mohammad Gharravi Shahroud University of Medical Sciences, Shahroud, Iran

Mohammad Reza Gholami Kermanshah University of Medical Sciences, Kermanshah, Iran

M. O. Gomzikova Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

Alberto González-González Grupo de Ingeniería de Tejidos de Cantabria, Dpto. Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IDIVAL, Santander, Cantabria, Spain

Kavitha Govarthanan MK BHAN Young Research Fellow, Centre for Cardiovascular Biology and Disease, Institute for Stem Cell Sciences and Regenerative Medicine (inStem), Bengaluru, Karnataka, India

Giulia Grisendi Division of Oncology, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy

Changfa Guo Department of Cardiac Surgery, Zhongshan Hospital, Fudan University, Shanghai, People's Republic of China

Piyush Kumar Gupta Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, Uttar Pradesh, India

Department of Biotechnology, Graphic Era Deemed to be University, Dehradun, Uttarakhand, India

Khawaja Husnain Haider Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia

Carl Randall Harrell Regenerative Processing Plant, LLC, Palm Harbor, FL, USA

Abdelkrim Hmadcha Department of Biotechnology, University of Alicante, Alicante, Spain

University of Pablo de Olavide, Sevilla, Spain

Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid, Spain

V. James School of Veterinary Medicine and Science, University of Nottingham, Nottingham, UK

Marc G. Jeschke Sunnybrook Research Institute, Toronto, ON, Canada

Division of Plastic Surgery, Department of Surgery, University of Toronto, Toronto, ON, Canada

Department of Immunology, University of Toronto, Toronto, ON, Canada

Ross Tilley Burn Centre, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

Mohamed Kamal Pharmacology and Biochemistry Department, Faculty of Pharmacy, The British University in Egypt (BUE), Cairo, Egypt

Biochemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Faculty of Pharmacy, Center for Drug Research and Development, The British University in Egypt (BUE), Cairo, Egypt

Dina Kassem Biochemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Mohamed Abdelghafour Khalifa Sulaiman AlRajhi Medical School, Al Bukairiyah, Kingdom of Saudi Arabia

Shahid Y. Khan The Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Mohamad Khazaei Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada

Eun Hee Kim S&E Bio, Inc, Seoul, South Korea

Oleg Knyazev Department of Inflammatory Bowel Diseases, Moscow Clinical Scientific Center Named After A. S. Loginov, Moscow, Russia

Department of Inflammatory Bowel Diseases, State Scientific Centre of Coloproctology Named After A. N. Ryzhih, Moscow, Russia

Mikhail Konoplyannikov Federal Research Clinical Center of Specialized Medical Care and Medical Technologies of the FMBA of Russia, Moscow, Russia

Department of Advanced Biomaterials, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia

Jan Lakota Centre of Experimental Medicine, Slovak Academy of Sciences, Bratislava, Slovakia

Faculty of Management, Comenius University, Bratislava, Slovakia

Danlin M. Law Cell Therapy Institute, Wuhan, China

Daniel Law Jia Xian Centre for Tissue Engineering and Regenerative Medicine (CTERM), Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Jalan Yaacob Latif, Kuala Lumpur, Malaysia

Peter K. Law Cell and Gene Therapies, Cell Therapy Institute, Wuhan, China

Department of Oncology, Renmin Hospital of Wuhan University, Wuhan, China

Qiaoyun Li Zhengzhou University, Henan, China

Wenbin Li Capital Medical University, Beijing, China

Lisa Lin Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada

Dingqian Liu Department of Cardiac Surgery, Zhongshan Hospital, Fudan University, Shanghai, People's Republic of China

Qi Hao Looi Future Cytohealth Sdn Bhd, Kuala Lumpur, Malaysia

Ping Lu Cell Therapy Institute, Wuhan, China

Jian-Hua Ma Nanjing Medical University, Nanjing, China

A. G. Malanyeva Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

Valentina Masciale Division of Thoracic Surgery, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy

William Brett McIntyre Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada

Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada

Mohamed Mekhemar Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian-Albrecht's University, Kiel, Germany

Ayşegül Mendi Faculty of Dentistry Department of Basic Sciences, Gazi University, Ankara, Turkey

Gyeong Joon Moon AMC Stem Cell Center, Asan Medical Center, Seoul, South Korea

Department of Convergence Medicine, University of Ulsan College of Medicine, Seoul, South Korea

Bihter Muratoğlu Center for Stem Cell Research and Development (PEDI-STEM), Hacettepe University, Ankara, Turkey

Institute of Health Sciences, Department of Stem Cell Sciences, Hacettepe University, Ankara, Turkey

Mustapha Najimi Laboratory of Pediatric Hepatology and Cell Therapy, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium

Min Hwei Ng Centre for Tissue Engineering and Regenerative Medicine (CTERM), Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Jalan Yaacob Latif, Kuala Lumpur, Malaysia

Kinglsey Chinonyerem Nnawuba Department of Clinical Medicine, School of Medicine, Caribbean Medical University, Willemstad, Curaçao

Edward Hosea Ntege Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan

Tom Ollila Division of Hematology and Oncology, Rhode Island Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, USA

Adekunle Ebenezer Omole Department of Anatomical Sciences, American University of Antigua College of Medicine, St. John's, Antigua and Barbuda

Yash Parekh CSIR–Centre for Cellular and Molecular Biology, Hyderabad, India

Ari Pelcovitz Division of Hematology and Oncology, Rhode Island Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, USA

Flor M. Pérez-Campo Grupo de Ingeniería de Tejidos de Cantabria, Dpto. Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IDIVAL, Santander, Cantabria, Spain

Katarzyna Pieczonka Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada

Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada

John S. Pixley Texas Tech University Health Science Center, Lubbock, TX, USA

Elio A. Prieto Gonzalez Centre for Advanced Studies in Humanities and Health Sciences, Interamerican Open University, Buenos Aires, Argentina

ISALUD University, Nutrition Career, Buenos Aires, Argentina

Peter Queseneberry Division of Hematology and Oncology, Rhode Island Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, USA

Israa Ahmed Radwan Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Dina Rady Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

M. Rajasundari Centre for Advanced Research (Stem Cells), Government Stanley Hospital, Chennai, Tamil Nadu, India

Jun Ren Tong Ren Hospital of Wuhan University, Wuhan, China

Mathieu Rheault-Henry Division of Cardiac Surgery, Health Sciences North, Northern Ontario School of Medicine, Sudbury, ON, Canada

S. Amer Riazuddin The Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Domenico Ribatti Department of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy

A. A. Rizvanov Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

José C. Rodríguez-Rey Grupo de Ingeniería de Tejidos de Cantabria, Dpto. Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IDIVAL, Santander, Cantabria, Spain

C. S. Rutland Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Science, University of Nottingham, Nottingham, UK

Khadiga Mostafa Sadek Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Biomaterials Department, Faculty Dentistry, Cairo University, Cairo, Egypt

António J. Salgado Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

Maurilio Sampaolesi Translational Cardiomyology Laboratory, Stem Cell and Developmental Biology, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Histology and Medical Embryology Unit, Department of Anatomy, Histology, Forensic Medicine and Orthopaedics, Sapienza University of Rome, Rome, Italy

Nihal Satyadev Department of Anatomical Sciences, University of Medicine and Health Sciences, Basseterre, Saint Kitts and Nevis

Jose Miguel Sempere-Ortells Department of Biotechnology, Immunology Division, University of Alicante, Alicante, Spain

Vineeta Sharma Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamilnadu, India

Qizhong Shi Xinxiang Medical University, Henan, China

Yusuke Shimizu Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan

Rita Caridade Silva Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

Tarik Smani Cardiovascular Pathophysiology, Institute of Biomedicine of Seville (IBiS), University Hospital of Virgen del Rocío/University of Seville/CSIC, Seville, Spain

Department of Medical Physiology and Biophysics, University of Seville, Seville, Spain

Biomedical Research Networking Centers of Cardiovascular Diseases (CIBERCV), Madrid, Spain

Tarik Smani Cardiovascular Pathophysiology, Institute of Biomedicine of Seville, University Hospital of Virgen del Rocío/University of Seville/CSIC, Seville, Spain

Qibin Song Renmin Hospital of Wuhan University, Wuhan, China

Shi Jun Song Xinxiang Medical University, Henan, China

Bernat Soria University of Pablo de Olavide, Sevilla, Spain

Department of Physiology, Institute of Bioengineering-Alicante Institute for Health and Biomedical Research (ISABIAL), University Miguel Hernández School of Medicine, Alicante, Spain

Li-ping Su Biomedical Engineering, University of Alabama at Birmingham, Birmingham, Alabama, USA

Nadiah Sulaiman Centre for Tissue Engineering and Regenerative Medicine (CTERM), Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Jalan Yaacob Latif, Kuala Lumpur, Malaysia

Hiroshi Sunami Center for Advanced Medical Research, School of Medicine, University of the Ryukyus, Okinawa, Japan

Renata Szydlak Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland

Roberto Tamma Department of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy

Yong Sheng Tan Cardiothoracic Department, Serdang Hospital, Kajang, Selangor, Malaysia

Fábio G. Teixeira Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

ICVS/3B's – PT Government Associate Laboratory, Braga/Guimaraes, Portugal

Juan R. Tejedo Department of Molecular Biology and Biochemical Engineering, University of Pablo de Olavide, Sevilla, Spain

Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid, Spain

Glen F. Tibbits Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada

Cellular and Regenerative Medicine Centre, BC Children's Hospital Research Institute, Vancouver, BC, Canada

Peter Timashev Department of Advanced Biomaterials, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia

Laboratory of Clinical Smart Nanotechnologies, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia

Department of Polymers and Composites, N. N. Semenov Institute of Chemical Physics, Moscow, Russia

Chemistry Department, Lomonosov Moscow State University, Moscow, Russia

Johannes Tölle Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian-Albrecht's University, Kiel, Germany

Duygu Uçkan-Çetinkaya Faculty of Medicine, Department of Pediatrics, Division of Hematology, Hacettepe University, Ankara, Turkey

Center for Stem Cell Research and Development (PEDI-STEM), Hacettepe University, Ankara, Turkey

Institute of Health Sciences, Department of Stem Cell Sciences, Hacettepe University, Ankara, Turkey

Roohi Vinaik Sunnybrook Research Institute, Toronto, ON, Canada

Ana Volarevic Department of Cognitive Psychology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

Vladislav Volarevic Department of Genetics and Department of Microbiology and immunology, Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

Jing Wang Renmin Hospital of Wuhan University, Wuhan, China

Keqiang Wang Xinxiang Medical University, Henan, China

Ian White Division of Cardiac Surgery, Health Sciences North, Northern Ontario School of Medicine, Sudbury, ON, Canada

Darius Widera Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, University of Reading, Reading, UK

Manye Yao Children's Hospital Affiliated to Zhengzhou University, Henan Children's Hospital, Zhengzhou Children's Hospital, Zhengzhou, China

Lei Ye Biomedical Engineering, University of Alabama at Birmingham, Birmingham, Alabama, USA

Laura Yedigaryan Translational Cardiomyology Laboratory, Stem Cell and Developmental Biology, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

E. Zakirova Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

Robert Chunhua Zhao Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China

Department of Cell Biology, School of Life Sciences, Shanghai University, Shanghai, China

Binita E. Zipporah Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamilnadu, India

Part I

Cell-Based Therapy Approach: Mesenchymal Stem Cell-Based Therapy

Human Mesenchymal Stem Cells: The Art to Use Them in the Treatment of Previously Untreatable

1

Jan Lakota, Maria Dubrovcakova, and Khawaja Husnain Haider

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Abstract

Mesenchymal stem cells (MSCs) can be isolated from almost all organs and tissues in the human body. For practical purposes, there are two main sources for their isolation and ex vivo expansion – the bone marrow and fat tissue. Based on their inherent plastic adherence properties, the ex vivo expansion of

J. Lakota (✉)
Centre of Experimental Medicine, Slovak Academy of Sciences, Bratislava, Slovakia

Faculty of Management, Comenius University, Bratislava, Slovakia
e-mail: jan.lakota@savba.sk

M. Dubrovcakova
Biomedical Center, Slovak Academy of Sciences, Bratislava, Slovakia
e-mail: exonmadu@savba.sk

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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MSCs is a rather simple process. Nevertheless, the biological features (gained from decades of tissue culture experience) are contrary to bureaucratic rules, which govern the good laboratory practice. MSCs cannot be successfully used in the treatment of human diseases if they are not handled optimally akin to the conditions in their natural habitat. Moreover, extrapolation of the data obtained from animal studies (mainly rodents) to humans is unfounded and with little relevance. The therapeutic use of genetically manipulated MSCs in human can be even harmful to patients. The current research paradigm, i.e., the use of MSCs in advanced phases of clinical trials although understandable, is far from personalized medical approach. The use of allogeneic versus autologous MSC in the clinical perspective is still debatable. There is growing evidence that the autologous MSCs derived from sick patients are "ill" in contrast to MSCs derived from healthy allogeneic donors. One can observe various changes at the DNA, RNA, and protein level in these "ill" cells. However, the huge number of cells from ex vivo expanded autologous MSCs can, possibly, overcome these aberrations. The off-the-shelf availability of allogenic MSCs also contributes to their logistic superiority over autologous cells. Moreover, due to almost non-existing immunological barriers, allogenic MSCs are emerging as gold standard and near-optimal cell types for the treatment of various diseases in humans. This chapter reviews the authors experience(s) in the treatment of various diseases with autologous/allogenic MSCs handled optimally ex vivo.

Keywords

Adipose tissue · Allogeneic · Autologous · Bone marrow · Ex vivo · Good biological praxis (GBP) · Human disease · MSCs · Treatment

List of Abbreviations

DMEM-LG	Dulbecco's modified Eagle medium, low glucose
GMP	Good Manufacturing Practices
GVDH	Graft-versus-host disease
LG	Low glucose
LVAD	Left ventricular assist device
MSCs	Mesenchymal stem cells
NYHA	New York Heart Association

Introduction

The paper of Koç (Koç et al. 2000) symbolically "opened the door" for the mesenchymal stem cells (MSCs) in the third millennium. Here, the authors reported about the autologous blood stem cells and in tissue culture (in vitro or ex vivo) expanded bone marrow-derived MSCs in advanced breast cancer patients receiving high-dose chemotherapy. Since then, an enormous amount of material has been published (Musiał-Wysocka et al. 2019). Coming down to the molecular level, our

knowledge each day is growing exponentially. Nevertheless, the primary question remains: What is the "therapeutic mechanism" of the applied MSCs? We call this effect as "posthypnagogic command." After the treatment with MSCs, the effect of healing is present for months, without the proven presence of MSCs. We are not coming in detail here; the reader could educate himself in the enormous amount of literature.

Mesenchymal Stem Cells Current Status: The "Problems" That in Reality Do Not Exist

Practical Approaches: The Golden Standards, i.e., "Good Biological Praxis" (GBP) Using the Historical Laboratory Experience

In our opinion, it is useful to repeat the whole procedure of ex vivo expansion in detail as it has been described in part "*Ex Vivo* MSCs Culture" (Koç et al. 2000): "Mononuclear cells (from bone marrow) were re-suspended at 10^6 cells/mL in Dulbecco's modified Eagle medium, low glucose (DMEM-LG) with 10% fetal bovine serum and 30 mL of cell suspension was plated in a 175 cm^2 flask. MSCs were cultured in humidified incubators with 5% CO_2 and initially allowed to adhere for 72 h, followed by media change every 3–4 days. When cultures reached more than 90% confluence, adherent cells were detached with 0.05% trypsin-EDTA." Later, additional characterizations and refinement added some regulatory rules. These ex vivo expanded MSCs fulfilled the criteria provided (later) by the International Society for Cellular Therapy (Horwitz et al. 2005). Briefly, MSCs are defined by their plastic-adherent properties under standard culture conditions, by their ability to differentiate into osteocytes, adipocytes, and chondrocytes in vitro under a specific stimulus and by positive (CD105, CD73, and CD90) or negative (CD45, CD34, CD14, and HLA-DR) expression of specific surface markers. There are two main sources for their isolation and ex vivo expansion for practical purposes – the bone marrow and the fat tissue. This ex vivo expansion of MSCs is a rather simple process based on their inherent plastic adherence properties. The pilot paper by Le Blanc et al. (2004) described the use of "third party" (here – haploidentical) MSCs for transplantation in a patient with severe treatment-resistant grade IV acute graft versus host disease (GVHD) of the gut and liver after allogeneic stem cell transplantation. For decades, two organs (or tissues), i.e., bone marrow and fatty tissue, were the primary sources for the isolation and ex vivo expansion of MSCs. The task of using allogeneic MSCs (obtained from healthy donors) or autologous MSCs in clinical settings will be discussed later.

MSCs cannot be successfully used to treat human diseases if they are not handled optimally akin to the conditions in their natural habitat. Let us discuss this in depth. As an example, we will consider the research paper published by Yau and colleagues (Yau et al. 2019). The authors claimed that "among patients with advanced heart failure, intramyocardial injection of mesenchymal precursor cells, as compared with the injections of a cryoprotective medium as sham treatment, did not improve successful temporary weaning from left ventricular assist device (LVAD) support

at 6 months. These findings do not support the use of intramyocardial MSCs to promote cardiac recovery as measured by temporary weaning from device support." According to the authors, the patients were randomly assigned to cell therapy group who received intramyocardial injection of 150 million MSCs and a cryoprotective medium treatment group without cells for comparison. The allogeneic MSCs were obtained from healthy donors and expanded in a Good Manufacturing Practices (GMP) certified laboratory. It is evident that the cells were thawed directly before use ("injections of mesenchymal precursor cells, compared to injections of a cryoprotective medium as sham treatment"). The cells were neither washed nor cultivated further for expansion before use. In the opinion of the authors that it is mandatory to use the MSCs that have been freshly prepared and not frozen or thawed immediately before use.

After decades of expanding the MSCs (and other cells) ex vivo (in vitro), we firmly stand behind this point of view. After thawing, the cells need to be cultured at least for 48 h in humidified incubators supplemented with 5% CO_2. Only after this wait period, one should start to consider further experimental (or therapeutic) work using these cells. On the other hand, one can consider growing the cells ex vivo, detaching them when 80% confluent and applying them to the patient in a short time (up to 3 h at room temperature). The practice to use freshly thawed cells (MSCs) makes the abovementioned study (and others in this fashion designed trials) from the biological point of view rather dubious and medically useless.

It should be noted that the number of skeptical articles and comments about the relevance of the MSCs for cell-based therapy is growing (Gomez-Salazar et al. 2020; Curfman 2019). We strongly disagree with the emerging notion. In our opinion, it is necessary to return to the laboratory and to give the MSCs a "second chance" by consequently following the GBP developed during the decades of cell tissue culturing in vitro. We recommend returning to the praxis of small tissue culture centers associated with (or localized within) the hospitals. In coordination with the hospital departments, they could prepare fresh MSCs, which would be "on demand" prepared for use and treat the patients. Logistically, to prepare a total of 20–50 $\times$ 10^6 cells is not a difficult task. One skilled technician could obtain this amount under sterile conditions in 1–2 h. What about the tests for the differentiation and of sterility? Well, yes, one can ask a heretical, unorthodox question: Did anybody ever observe that the MSCs in vitro did not differentiate to osteoclasts, adipocytes, and chondrocytes during appropriate treatment? This has been further discussed elsewhere in the chapter.

Data Extrapolation from Small Animal Studies to Humans Is Unfounded and Without Relevance

The therapeutic use of genetically manipulated MSCs in humans is even harmful to patients. The engineered ("therapeutic") MSCs are genetically modified MSCs that contain a stable gene encoding for protein or enzyme product/s able to kill the tumor cells. A typical example of such a construct is the yeast enzyme cytosine deaminase,

which converts the rather nontoxic 5-fluorocytosine to the cytostatic agent 5-fluorouracil (Kucerova et al. 2007). The results obtained from rodents, i.e., preclinical experimental models, are promising. In a recently published review article, Pawitan and colleagues have stated: "So far, most studies using pre-clinical cancer models have shown consistent results, i.e., the engineered MSCs could inhibit tumor growth and enhance the survival rate of the tumor-bearing animals" (Pawitan et al. 2020). And only one published clinical paper by Lakota et al. (2015) claims the opposite: "Treatment with therapeutic MSCs (i.e., genetically engineered MSCs) of this patient highlighted the following points" (Table 1):

1. There was no evidence of any therapeutic benefit after intravenous administration (not local, i.e., intra-tumoral injection) of the therapeutic MSCs. Six days after the cell administration, the metastatic process did not show any signs of regression. Moreover, 40 days after the treatment, there was a progression of the metastases.
2. After the intravenous administration, the therapeutic MSCs were probably "homing" into the bone marrow despite their adipose tissue of origin. Even a relatively low cell count ($60x10^6$) was able to cause grade 2 (resp. grade 3) thromobocytopenia (resp. neutropenia)

It should be noted that this patient did not receive any systemic chemotherapy in the past. The observed bicytopenia with a nadir neutropenia occurred 48 h after administering therapeutic MSCs (with concomitant prodrug administration). Moreover, in a more recently published paper by Lakota (2018), the author claims that:

(i) There was no sign of any therapeutic effect after intravenous administration (not local, i.e., intra-tumoral) of the "therapeutic" MSCs.
(ii) After the intravenous administration, the "therapeutic" MSCs were "homing" into the bone marrow.
(iii) There has not been any entrapment of the "therapeutic" MSCs in the lungs (after the intravenous administration).

Table 1 Blood counts of the patient with head and neck tumor during and after the therapy with therapeutic MSCs. (The table is taken from Lakota et al. 2015)

Day	Leukocytes ($\times10^{-12}$/l)	Neutrophils ($\times10^{-12}$/l)	Erythrocytes ($\times10^{-15}$/l)	Hb (g/l)	Plt ($\times10^{-14}$/l)
−2	6.88	4.86	4.41	134	179
0	5.82	3.98	3.71	119	150
+1	4.06	3.41	3.80	119	119
+2	1.99	1.00	3.41	112	100
+3	2.89	1.58	3.72	117	115
+4	3.50	2.16	3.72	115	122
+5	4.50	2.98	3.79	118	129
+6	4.29	2.63	3.89	121	132
+18	6.57	4.66	4.06	125	191

(iv) After local (i.e., in situ) administration, the "therapeutic" MSCs did not migrate to any "neighboring" tissue/organ (the liver, retroperitoneum, abdominal wall) or other distant organs, including the bone marrow.

Thus, the data obtained from small experimental animal models (mice and rats) must not be extrapolated to the humans. Moreover, the unrestrained transfer (although understandable) of the requisitioned data obtained with the MSCs from rodents may negatively influence the research trends in the novel treatment(s) of human diseases. This kind of treatment of human patients is ineffective and can be harmful and dangerous. The systemically administered therapeutic MSCs are rather homing into the patients' bone marrow and not into the tumor tissues. After local application (in the tumor area), their ability to destroy the whole tumor or its metastasis is limited. Nor they are moving to other metastatic tumor localizations.

Current Research Paradigm: MSCs in Advanced Phases of Clinical Trials Although Understandable Is Far from Personalized Medicine

Current praxis is treating human diseases based on evidence-based medicine (Masic et al. 2008, and the Internet). Clinical trials are the cornerstone to assess novel disease treatment(s), and among the clinical trials, randomized clinical trials are considered as the most preferred design as it provides a causal relationship between the medical intervention and the desired effects (Sawchik et al. 2018). A pertinent question here is: Is the same approach valid for MSCs which is fast emerging as a biopharmaceutical? Here we present some of our (published and unpublished) data none of which has been acquired in any registered trial. All of these data were obtained according to the rules of personalized medicine.

(i) Autologous bone marrow-derived MSCs were used for transplantation in ten patients with ischemic cardiomyopathy (Lakota 2014a). All patients had a well-documented history of anterior wall acute myocardial infarction. They were in NYHA stage III or IV. The freshly prepared MSCs were injected into the left anterior descending coronary artery. All of the patients tolerated the MSCs injection well and were discharged from the hospital 3–4 days after the procedure. Six patients died 7, 9, 37, 71, 101, and 119 months after the procedure. Four patients were alive 112, 113, 120, and 121 months after the MSCs-based treatment.

(ii) The allogeneic adipose tissue-derived MSCs were used for the treatment of the patient with 11 years history of ulcus cruris on his left leg, which remained stationary despite trials of conservative treatment (Lakota 2014b). The adipose tissue-derived MSCs (30×10^6) were applied locally (circumferentially). After a single round of MSCs treatment, the ulcus healing progressed "normally" despite repeated courses of standard and high-dose chemotherapy for patient's oncological diagnosis (high-grade non-Hodgkin's lymphoma). The final effect, i.e., restitutio ad integrum, has been observed during 6 months after

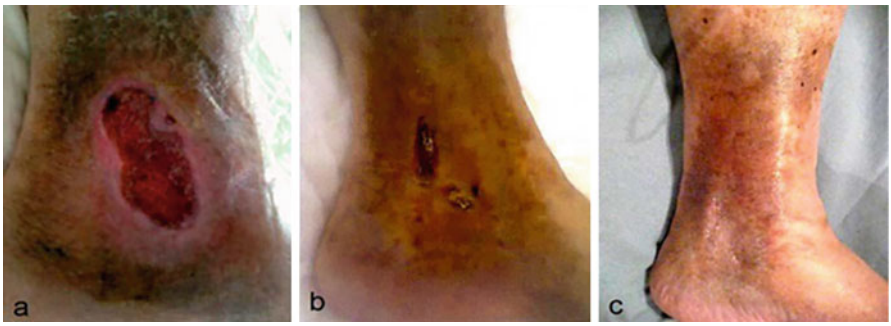

Fig. 1 Healing of the ulcus cruris after local application of MSCs. (**a**) 11/2012, day after application; (**b**) 02/2013, d + 25 after autologous stem cell transplantation; and (**c**) 05/2013. (The figure is taken from Lakota (2014b))

the MSCs-based treatment (Fig. 1). This course of healing did not differ from the patients with ulcus cruris who received allogeneic or autologous MSCs and who did not receive any chemotherapy (Lakota, unpublished).

(iii) Ten patients after allogeneic stem cell transplantation (high-dose therapy for hematological malignant disease) and with severe steroid-resistant grade IV acute GvHD were treated with allogeneic bone marrow-derived MSCs (range 0.3–0.5 $\times$ 10^6 MSCs/kg body weight) (Lakota 2017a). GvHD was successfully resolved in four patients who received MSCs-based therapy (Table 2). In the end, the author claimed: "Moreover, it seems plausible to have the MSCs 'in stock' for fast *ex vivo* expansion to use the freshly prepared cells (rather than frozen, thawed, and immediately used)."

(iv) The autologous bone marrow-derived MSCs and allogeneic adipose tissue-derived MSCs were used to treat aseptic necrosis of the jaw. The treatment was performed in 30 patients over a period of 15 years. Freshly prepared MSCs (20×10^6) from the respective tissues were applied locally. In five patients, MSCs were used twice. The result was a restitutio ad integrum (Fig. 2) (Lakota, unpublished data and Lakota 2017b, in Slovak).

(v) The allogeneic adipose tissue-derived MSCs were used for the treatment of ten patients with sclerosis multiplex. The target dose has been 0.5 $\times$ 10^6 cells/kg bodyweight. The patients were advised not to stop current "official" treatment. Freshly prepared MSCs were delivered intravenously. No adverse effects were observed. The CNS lesions in all patients but one did not progress during 15 years of follow-up after MSCs-based cell therapy (Lakota, unpublished data).

We did not mention all the data obtained by the authors during the last two decades involving MSCs-based cell therapy. The authors never performed any double-blind randomized clinical trials due to logistic and economic reasons. In our hands, personalized medicine and the medical treatment followed the golden rule: "primum non nocere." We followed the aim and principle of helping the sick

Table 2 Patients' characteristics, date of transplantation, date of MSCs infusion, date of the patients' death, and the reason of death. (Haplo Tx: HLA haploidentical transplantation; RI Tx: reduced intensity conditioning transplantation). (The table is taken from Lakota 2017a)

Patient No	Gender	Born	Diagnosis	Transplantation	MSC infusion	Death	Reason of death	Comment
1	M	1971	AML	16.03.04	d + 206	09.11.04	cGvHD	
2	M	1976	HD	30.01.05	d + 1	14.02.05	aGvHD	Haplo Tx
3	M	1971	ALL	13.12.05	d + 9	05.04.06	Disease progression	
4	F	1970	Ph-CML	26.04.07	d + 33	–	Alive (31.12.16)	
5	F	1978	HD	13.10.09	d + 60, d + 80	23.02.10	aGvHD	RI Tx
6	M	1969	ALL	23.02.10	d + 168	16.08.10	cGvHD	
7	F	1973	HD	21.10.10	d + 92, d + 178	22.09.11	Infection	HaploTx
8	M	1987	HD	18.10.11	d + 43	09.04.13	Disease progression	RI Tx

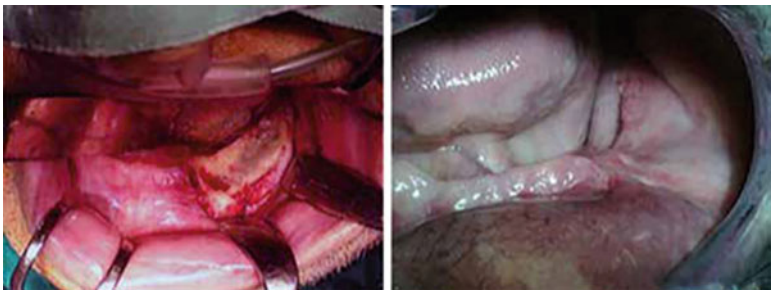

Fig. 2 Healing of the osteonecrosis of the jaw after local application of MSCs. Left: during the surgery; right: 12 weeks after the surgery. (The figure is taken from Lakota 2017b)

and suffering patients in concert to see how serpentine can be the road to establish a new treatment in medicine. It is worth mentioning the following observation. In 2004, Vulliet and colleagues published an interesting research paper of using MSCs in a healthy dog (Vulliet et al. 2004). The authors delivered 0.5 million MSCs/kg bodyweight into the canine left circumference coronary artery. The authors observed ECG changes (ST-segment elevation) in all dogs receiving cell administration which was a characteristic of acute myocardial ischemia. Besides microscopic and macroscopic evidence of myocardial ischemia, the authors also observed increased troponin I in two dogs in which measurements were made. These data suggested that myocardial ischemia occurred after the injection of MSCs at the dose used in this study. Microinfarction was also confirmed with histological and immunocytochemical data.

These results showed a potential complication of injecting MSCs, or probably any similarly sized cell, into the coronary circulation. Although differences between canine and human coronary circulation exist, and different cell types and sizes have been used for selected cytotherapeutic applications, this potential complication should be thoroughly investigated before MSCs are routinely injected into the arterial circulation of the patients. Luckily for us (Lakota 2014a) and for the patients at the time of publishing this paper (Vulliet et al. 2004) as we became aware of that we performed three intracoronary MSCs transplants and three other patients were "on the horizon." Thus, this is another confirmation of how dubious the translation of "(pseudo)clinical" data obtained on animals to human clinics is. Two recently published reviews in this regard have elegantly discussed the donor-related factors and quality of the cell preparation as the possible determinants of the outcome of cell-based therapy in the clinics (Haider 2018; Rady et al. 2020).

A Case of Autologous Versus Allogeneic MSCs, an Unanswered Question

One of the ongoing and inconclusive debates in cell-based therapy is the preference for autologous over allogeneic MSCs. However, the use of allogeneic MSCs for

cell-based therapy is fast emerging as a new paradigm in therapeutics (Karantalis et al. 2016). Starting with the pioneering work of Orlic et al. (2001), preclinical studies have characterized syngeneic, xenogenic, allogeneic, and autologous MSCs; however, most of these studies, especially in the small animals, i.e., mice, rats, etc., have focused on the use of allogenic MSCs due to ease of availability (Orlic et al. 2001, Fukuda and Fujita 2005; Jiang et al. 2006; Haider et al. 2008; Beitnes et al. 2012). Similarly, in large animal translational studies, autologous and allogeneic MSCs have been used without any safety issues with either cell type (Poh et al. 2007; Quevedoa et al. 2009; Chen et al. 2014). But then the question remains why these data have been ignored while designing the clinical trials wherein mostly autologous MSCs have been focused on cell-based studies.

As evidenced by a systematic review and meta-analysis of 82 animal studies involving 1482 animals that both autologous and allogeneic cells are equally effective (Jansen of Lorkeers et al. 2015), but they have their respective advantages and limitations in the clinical perspective. Hence, the clinical researchers should give these parameters due consideration during clinical study design for optimal therapeutic outcomes. The following section discusses in depth the pros and cons of each cell source in the light of the published data and implores that the relevant information should be given due consideration, especially during the design of clinical studies.

A Case for Autologous Cells

On its face, the fear of incompatibility is alleviated with the use of autologous cells. Hence, autologous cells' usage is advantageous as it does not require immunosuppression to support the acceptance of the transplanted cells and their derivative tissue after differentiation posttransplantation. Moreover, treatment with autologous cells is considered a step closer to the fast-emerging personalized medicine. However, on the downside of autologous cell-based therapy, the use of autologous cells is time-consuming and labor-intensive exercise, and clinically less viable option, as it may necessitate biobanking of autologous cells for future use of the cells for every individual. On the same note, it is also not sustainable for diseases where the patient may require early intervention, i.e., myocardial infarction, stroke, etc., and the patient does not have the choice of long waiting time until the harvested cells can be purified and expanded to achieve the required cell number for transplantation. Some essential considerations in case autologous cells are as follows:

Autologous Cells from Patients Are Also "sick"

There is growing evidence that the autologous cells derived from the patients eligible for the cell-based treatment are "sick" compared to the ones derived from healthy allogeneic donors due to their exposure to different risk factors and comorbidities in the "sick" donors (Dimmeler and Leri 2008; Cesselli et al. 2011). Moreover, it is now well-documented that many diseases compromise the stem cell niche homeostasis and seriously affect stem cell properties such that they are rendered unsuitable for cell-based therapy (Perez et al. 2018). For example, Liu et al. have shown that MSCs from diabetic patients have impaired cardioprotective function as compared to the

ones derived from the healthy donors, which was ascribed to the long-term exposure to hyperglycemia (Liu et al. 2013).

We have performed RNA microarray analysis comparing MSCs from two patients with ischemic cardiomyopathy and two healthy donors (Lakota 2014a). Data analyses showed a significantly enhanced gene expression ratio for STAT1α/β (signal transducer and activator of transcription 1-alpha/beta) and ISG15 (ISG15 or g1p2 or ucrp; ubiquitin cross-reactive protein). The decreased ratio of gene expression has been shown for GTP-binding protein RAD. The first analyzed patient died 101 months after the procedure. The second one was alive 120+ months after the procedure.

It should be noted that the MSCs in all patients treated were isolated from their bone marrow. This data clearly shows that ischemic cardiomyopathy is a "systemic" disease. One can speculate about the following fact: How is it possible that the patients with this "damaged" RNA profile in MSCs survived such a long time after the procedure, i.e., such "sick" MSCs were able to repair the damaged myocardium?

Autologous Cells in Elderly Patients Are Also Aged

Another critical aspect generally overlooked during the use of autologous cells is the donor age besides the age of the recipient. A considerable majority of the patient population who are candidates for cell-based therapy are elderly. Using their own (autologous) cells for cell-based therapy and transplanting them back into an aging tissue environment (which has lost the vigor of reparability due to chronological aging) accounts to double negative that significantly hampers the therapeutic outcome (Zhuo et al. 2010).

It is pertinent to mention that similar to any other body cell, stem cells also undergo chronological aging, which is multifactorial, i.e., metabolic alteration, accumulation of reactive oxygen species, accumulation of DNA damage and mutations, telomere shortening, etc. (Oh et al. 2014; Schultz and Sinclair 2016). These metabolic and molecular-level changes lead to loss of their stemness characteristics with age, which is reflected in the impairment of their functionality (Kissel et al. 2007; Bustos et al. 2014). Moreover, chronological aging also leads to a significant reduction in stem cells (Maijenburg et al. 2012). Stenderup et al. carried out a direct in vitro comparison of bone marrow-derived MSCs from young donors (18–29-year-old, $n = 6$) versus elderly donors (68–81-year-old, $n = 5$) (Stenderup et al. 2003). The authors compared the cells for the expression of senescence markers, cell growth, and differentiation potential. It was observed that the cells from elderly donors showed significantly reduced maximal life span in terms of population doublings (PD) and PD rate, and accelerated senescence as evidenced by beta-galactosidase expression as a marker. Similarly, a comparison of bone marrow-derived MSCs from human donors (17–90 years age) showed a significant increase in doubling time beside increased expression of senescence markers with the advancing age of the donor (Zhou et al. 2008).

Chronological aging and obesity also cause a decline in stem cell yield and their ability to hematopoiesis and bone regeneration (Pachon-Pena et al. 2016, Ambrosi et al. 2017). Therefore, it is essential that clinical researchers consider the

consequence of donor age while opting for autologous cell sources (Stolzinga et al. 2008). A direct comparison of the reparability of bone marrow-derived MSCs showed that the rate of old donor bone marrow-derived MSCs had poor cardiomyogenic differentiation potential as compared to the MSCs derived from young donor bone marrow (Jiang et al. 2008). For comparison, the cells were transplanted in the same heart in an experimental animal model of acute myocardial infarction. Khan et al. showed that the reparability of the senescent myocardium is determined by the age of the donor (Khan et al. 2011). All these data signify that MSCs from aging patients show a drastic loss of their biological activity and bring us to an important question if the use of autologous is the real culprit for the modest outcome of the clinical trials reported to date (Shahid et al. 2016).

A Case for Allogeneic Cells

Data emanating from the clinical studies have shown the safety and efficacy of allogeneic MSCs in adult and pediatric patients (Koc et al. 2002; Horwitz et al. 2002). Recent clinical application of allogeneic MSCs in ischemic cardiomyopathy patients vindicated these data and reported that allogeneic MSCs were as good as autologous MSCs in their functionality and efficacy, favorably affecting LV end-diastolic volumes, LVEF, and ventricular remodeling leading to improved quality of life (Hare et al. 2012, 2017). More importantly, these studies did not report any severe adverse reactions associated with the cell-based therapy with allogeneic cells, including immunologic responses. The safety profile of allogenic MSCs has also been substantiated during a systematic review and meta-analysis of 36 clinical studies, including 1012 participants (Lalu et al. 2012). Experimental studies assessing immunological profiling of MSCs have shown that although they are not immunopriviledged, allogeneic MSCs are weakly immunogenic, because they lack MHC class II and co-stimulatory molecules, i.e., CD40, CD80, and CD86, while they have weak MHC class I expression (Machado et al. 2013; Lohan et al. 2014). Moreover, they do show immunomodulation by suppressing the activation and proliferation of immune cells (Asari et al. 2009; Corcione et al. 2006). Their interesting immune profile tips them as a good candidate for cell-based therapy without the need for immunosuppression therapy and takes care of them not being "self" for the recipient (Kariminekoo et al. 2016). These data about the allogeneic MSCs are a step forward towards the ongoing quest for "Universal donor cells," which should be available off-the-shelf as a ready-to-use cell preparation (Kinkaid et al. 2010).

Logistic Advantage of Allogenic MSCs

One of the primary advantages of allogenic MSCs is their logistic superiority over autologous cells (Zhang et al. 2015). Unlike autologous MSCs, which need to be isolated, purified, and expanded in culture before use for each patient, allogenic cells are logistically feasible as they may be readily available off-the-shelf. This ready availability makes possible their use in urgent clinical situations, which is not possible with the autologous cells as it may take 3–4 weeks of isolation, purification,

and expansion before they could be used for delivery. For any cell-based therapy to be of routine clinical significance as a therapeutic modality, it is imperative that the cells must be available off-the-shelf akin to any other conventional pharmacological agent. Despite the fast-emerging innovative field of personalized medicine, drugs are not to be synthesized for each patient; instead, their use is tailored according to the need of the patients who are stratified to enhance therapeutic efficacy (Marshall et al. 2016).

Similarly, in cell-based therapy, which is one form of personalized medicine, cells cannot be prepared for each patient before use; there have to be readily available cell preparations, which can be tailored to the need of the patient. Manufacturing large clinical grade batches of allogenic cell products using GMP, quality controlled for viability, self-renewal, and stemness characteristics will be cost-effective, time-saving, less labor-intensive, commercially favorable, and clinically more relevant for reproducible outcome. Moreover, this off-the-shelf approach fits well with the current pharmaceutical practices, scale-up manufacturing, and may involve automation to make it efficient in manufacturing, thus having a better commercial potential than autologous cells (Malik and Durdy 2015). Additionally, allogeneic MSCs may allow repeated doses of the cells which may be more beneficial than one-time treatment (Poh et al. 2007).

Allogenic MSCs Overcome the Limitations of Aging and Sickness of Autologous Cells

As discussed earlier, autologous cells derived from elderly patients, especially those with multiple comorbidities, may not fetch the desired results. Availability of allogeneic cells from a young healthy may be a better option for cell-based therapy. In a recently published study, which was aimed to identify a set of donors and their donated cells' characteristics with predictive value for optimal osteoblastic differentiation, bone marrow-derived MSCs from 58 patients undergoing surgery for bone fracture were characterized for high osteogenic potential and low adipogenic potential (Kowal et al. 2021). The authors have reported a well-defined criterion that donor cells obtained from male donors, without a diagnosis of osteoporosis and containing a higher fraction of $CD146^{+}$ fraction of cells, together were predictors of high osteogenic potential. Such predefined criterion is also warranted for selecting MSCs for use in the patients who are candidates for cell-based therapy for other diseases.

We will not go into detail here (Lakota 2016); nevertheless, one possible explanation could be that the myocardial repair occurred because of the presence of a considerable amount of ex vivo expanded MSCs. The RNA microarray analysis reflects only the statistically up- or downregulation of specific genes. In another study, Koh and coworkers suggested that pluripotency and the secretion of trophic factors of the bone marrow-derived MSCs in amyotrophic lateral sclerosis patients were reduced in proportion to a poorer prognosis. This may suggest that allogeneic (bone marrow- or adipose tissue-derived) MSCs from healthy donors may be a better option for MSCs therapy in amyotrophic lateral sclerosis patients (Koh et al. 2012).

Quality Control of MSCs Preparation: A Prerequisite for Optimal Prognosis

Autologous or allogeneic MSCs are part of the primary prerequisite of quality of the MSCs preparation for use in the patients (Haider 2018) and remain a fundamental determinant of the outcome of any cell-based therapy procedure and its success (Haider 2017). It encompasses everything from percentage cell count and cell viability, their identification to proliferation and differentiation potential to paracrine action of the cells in the MSCs preparation to ensure that the cell preparation is the best compromise of all these properties. For example, the cells should have the proliferation capacity, but at the same time, unlimited proliferation will add the risk of tumorigenicity. Hence, validating MSCs preparation, both biologically and functionally, will ensure that the cells will do the needful, which is meant for post-engraftment in the clinical settings. Unfortunately, these critical aspects of quality control of the cell preparation in general, and functional assessment in particular, have been generally overlooked during the design of the clinical studies. This has seriously impacted the efficacy of the cells, thus significantly contributing to the modest outcome of the clinical trials in most cases.

For example, bone marrow-derived MSCs, both autologous and allogenic, require in vitro culturing for at least 3–4 weeks. Out from their natural habitat, they are exposed for so long to the unnatural biological environment, which is only partially emulating their natural habitat at best, is expected to alter their biological as well as functional characteristics significantly. All this leads to their senescence or aging in culture due to less than optimal culture conditions (Bonab et al. 2006; Jiang et al. 2017; Shen et al. 2018). Moreover, the long-term culture may render the cells devoid of their specific surface marker expression, i.e., CD29, CD44, CD90, and induce chromosomal instability (Furlani et al. 2009). Transplantation of these in vitro expansion showed no functional effect post-engraftment in experimentally infarcted myocardium. Although various strategies have been developed to recover the culture-induced senescence of cells in terms of their proliferation capacity (Koichi et al. 2011; Tan et al. 2021), the cells may become transformed to be tumorigenic depending upon the culture condition, thus becoming unsafe for cell-based therapy (Rosland et al. 2009; Wolf et al. 2009).

Similarly, the long-term culture of the cells becomes immunogenic due to the settlement of extraneous proteins from the culture medium. These ill effects of prolonged in vitro culture are most pronounced in 2D culture conditions. It is anticipated that the advanced 3D culture conditions in vitro will go a long way in alleviating the effects of cell culture-induced cell senescence due to its biomimetic properties (Hoch and Leach 2014; Jeger-Madiot et al. 2021).

Another typical example is the effect of cryopreservation of the cells in clinical settings. Although the current cryopreservation protocols are well-optimized and successfully preserve the biological and functional characteristics of the cryopreserved cells, some aspects of the technique require further refinement (Mamidi et al. 2012). Cryopreserved MSCs show altered immunomodulatory and therapeutic efficacy with a significant reduction in the number of viable cells. The rate of cell viability, as well as cell clumping, is also an important complication of

cryopreservation that may contribute to micro-occlusions after intra-arterial delivery of the cells as compared to the freshly isolated cells with cryopreservation (Cui et al. 2016). A recent study has reported that the cryopreserved successfully maintained their multi-lineage differentiation, immunomodulatory and anti-inflammatory properties, but they lose some of their stemness characteristics in a reversible fashion, which the cells could recover within 24 h in the culture after thawing (Antebi et al. 2019). Kaplan et al. have elegantly reviewed the effect of cryopreservation of the MSCs biological and functional characteristics (Kaplan et al. 2017).

Conclusion

In our opinion, the question of the use of autologous versus allogeneic MSCs remains unresolved. Autologous MSCs, although sick, are returning home after ex vivo expansion when they are used for cell-based therapy. Allogeneic healthy cells are here on demand. The immunological barriers do not (in practice) exist. According to the authors' experience, one should not be afraid to use them in all cases when there are doubts about the current availability of autologous MSCs. No single negative effect has been ever observed. Therefore, the summary of theoretical pros and cons cannot solve up to date this problem.

In conclusion, we would like to remind the reader of the old rule which governs the laboratory praxis. This is what we call "good biological praxis" (GBP). One should not create problems where they do not exist. Or, better, with humbleness, we should approach the divine principles in nature which we receive as gifts. With these gifts earlier hidden, we will be able to treat previously untreatable. MSCs are the current example. Human medicine is hotly waiting.

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2 Sources and Therapeutic Strategies of Mesenchymal Stem Cells in Regenerative Medicine

Mohamed Kamal, Dina Kassem, and Khawaja Husnain Haider

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Abstract

During the past decade, mesenchymal stem cells (MSCs) have made their mark as a potential weapon in regenerative medicine. Since their first isolation by Friedenstein in the late 1970s of the last century, MSCs have opened new avenues

M. Kamal (✉)
Pharmacology and Biochemistry Department, Faculty of Pharmacy, The British University in Egypt (BUE), Cairo, Egypt

Biochemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Faculty of Pharmacy, Center for Drug Research and Development, The British University in Egypt (BUE), Cairo, Egypt
e-mail: mohamed.kamal@bue.edu.eg

D. Kassem
Biochemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt
e-mail: dina_kassem@pharma.asu.edu.eg

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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in the field of regenerative medicine. The main fascination about MSCs lies in their ease of isolation and large ex vivo expansion capacity, as well as demonstrated multipotency and immunomodulatory activities. Basically, several reports have proved that MSCs isolated from different sources possess different characteristics and potentials. In addition, the mechanisms by which these cells can help regenerate tissues and treat several diseases have been proved to be far more complicated than ever thought of. Moreover, a growing body of research has revealed that the therapeutic effects of MSCs occur largely via paracrine signaling and secreted extracellular vesicles, which act as "signalosomes" controlling fundamental cellular functions in recipient cells. In this chapter, we will discuss how MSCs isolated from different sources such as bone marrow; the prototype MSCs, adipose tissue, and umbilical cord differ in their characteristics as potential sources of allogenic versus autologous cell therapy options. Besides, we will clarify the main documented mechanisms of action which MSCs play in regenerative medicine including their differentiation to tissues of mesenchymal versus non-mesenchymal lineages. Additionally, the immune-modulatory effects of MSCs will be discussed as an important arm in their therapeutic potential. Finally, we will discuss the potential of extracellular vesicles produced by MSCs as an emerging cell-free alternative to stem cells therapy.

Keywords

Adipose tissue · Bone marrow · Cell free · Exosomes · Mesenchymal stem cells · Stem cells · Umbilical cord blood · Wharton's jelly

Abbreviations

BM	Bone marrow
CM	Conditioned medium
EVs	Extracellular vesicles
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GMP	Good Manufacturing Practice
GvHD	Graft vs host rejection disease
HGF	Hepatocyte growth factor
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN-γ	Interferon-γ
ISCT	International Society for Cellular Therapy
MSCs	Mesenchymal stem cells
NK cells	Natural killer cells
PDL-1	Programmed death ligand-1
PGE2	Prostaglandin E2
PRP	Platelet-rich plasma
SVF	Stromal vascular fraction
TGF-β1	Transforming growth factor-β1

TNF-α	Tumor necrosis factor-α
UC	Umbilical cord
UCB	Umbilical cord blood
WJ	Wharton's jelly

Introduction

Mesenchymal Stem Cells

During the past decade, the field of mesenchymal stem cells (MSCs)-based research has witnessed huge progress due to unraveling many of their exceptional biological features and stemness characteristics, as well as the encouraging results of several preclinical and clinical studies. Thus, it is now widely accepted that MSCs could provide a revolutionary therapeutic intervention for various diseases (Batsali et al. 2013; Wang et al. 2012). MSCs were first reported in the early 1970s when a population of plastic-adherent, fibroblast-like, non-hematopoietic cells were isolated from bone marrow (BM) (Friedenstein et al. 1974). Later, in the early 1990s, based on their properties, the term "mesenchymal stem cell" was proposed (Caplan 1991). The term "mesenchymal" is derived from the word mesenchyme, which refers to loosely organized tissue during embryonic development broadly associated with connective tissues (Viswanathan et al. 2019). Its noteworthy here that the acronym "MSCs" has also been designated for "*multipotent mesenchymal stromal cells*" (Horwitz et al. 2005).

Nevertheless, although both terms have been commonly used interchangeably in the literature, it is pertinent to mention that the International Society for Cellular Therapy (ISCT), now called the International Society of Cell and Gene Therapy, has clarified through a couple of position statements that the term "mesenchymal stem cell" is not equivalent to "mesenchymal stromal cells." Moreover, the latter refers to a bulk population with notable immunomodulatory as well as secretory and homing properties (Horwitz et al. 2005; Viswanathan et al. 2019). They have suggested that the term "mesenchymal stromal cells" can be used to describe the heterogeneous bulk population of cells, which includes fibroblasts, myofibroblasts, and even a relatively small proportion of stem/progenitor cells, but excludes endothelial and hematopoietic cells (Viswanathan et al. 2019).

Notably, the ISCT has defined specific minimal criteria for the proper characterization of MSCs so that the research data from various research groups may be compared (Dominici et al. 2006). This criterion includes:

1. MSCs are plastic-adherent when maintained in well-defined and standardized culture conditions.
2. MSCs must express some mesenchymal cluster of differentiation (CD) surface markers like CD105, CD73, and CD90 and lack the expression of hematopoietic stem cell-specific markers like CD45, CD34, and CD14.

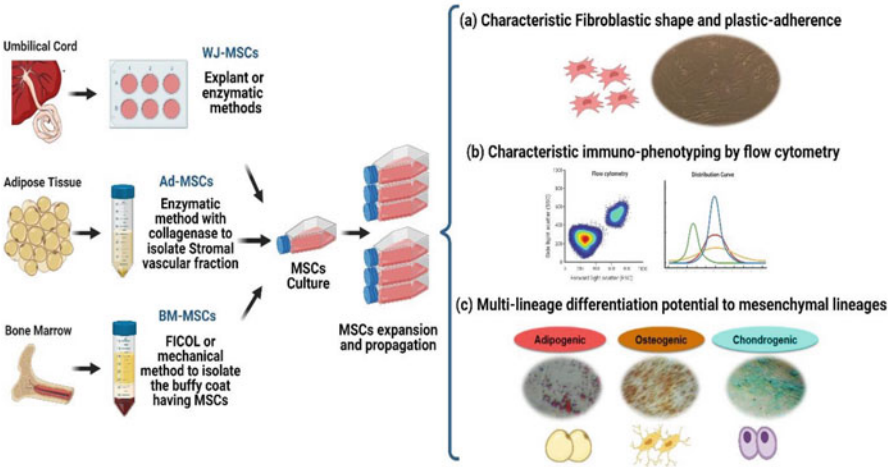

Fig. 1 Isolation and characterization of MSCs from different tissue sources, including umbilical cord, adipose tissue, and bone marrow. After isolation, MSCs from different tissue sources exhibit ISCT criteria based on plastic adherence, immunophenotyping, and trilineage differentiation potential of osteogenic, adipogenic, and chondrogenic phenotype. (Created with Biorender.com)

3. MSCs should exhibit multilineage differentiation potential to adopt adipogenic, osteogenic, and chondrogenic phenotypes in vitro in the presence of specific chemical cues.

These essential characterization criteria are summarized in Fig. 1.

Sources of Mesenchymal Stem Cells

So far, MSCs have been successfully isolated from various tissues, including adult tissues such as adipose tissue, BM, peripheral blood, dental pulp, skin, muscle, endometrium, ovary, as well as perinatal/fetal sources, like the placenta, umbilical cord blood (UCB), and umbilical cord (UC) matrix/tissue (Da Silva Meirelles et al. 2006; Jackson et al. 2010; Ma et al. 2014; Vasandan et al. 2014; Niezgoda et al. 2017; Berebichez-Fridman and Montero-Olvera 2018; Ghamari et al. 2021; Zolbin et al. 2021). Despite all of these being MSCs from different tissue sources, they significantly differ from each other in terms of their proliferation, self-renewal, paracrine action, and even differentiation potential. For example, MSCs obtained from UCB are rich in a more primitive cell population than those obtained from the BM (Hass et al. 2011; Wang et al. 2016b). Similarly, adipose tissue-derived MSCs (Ad-MSCs) have specific features, such as their transcriptome, proteome, and immunoregulatory properties as compared to the BM-derived counterparts (Strioga et al. 2012; Li et al. 2015a). Similarly, the contributing factors causing divergence in MSCs' characteristics include age, sex, the health status of the donor, etc.

For example, MSCs derived from a young donor (8–12 weeks) and elderly donor rats (24–26 months) was assessed for their responsiveness to anoxia and assessment reparability of infarcted myocardium post engraftment in an experimental animal model of acute myocardial infarction (Jiang et al. 2008). The young donor-derived cells were more resistant to apoptotic signals upon anoxic exposure than their counterparts. Similarly, the young donor-derived MSCs showed more cardiomyogenic differentiation after transplantation and better preserved the global cardiac function of the infarcted myocardium. Similar results have also been reported by Wang et al. while comparing young and old BM-derived MSCs in an experimental animal model for cardiac repair (Wang et al. 2008).

BM-derived MSCs have been the most extensively studied and denoted as the "gold standard" of MSCs among MSCs derived from different tissue sources (Arutyunyan et al. 2016). From among the most important tissues sources of MSCs, we will discuss in this chapter BM-derived MSCs, Ad-derived MSCs, and Wharton's jelly-derived MSCs (WJ-derived MSCs) with particular emphasis on the latter two as the most well-studied types of MSCs in regenerative medicine.

Bone Marrow-Derived MSCs

BM-derived MSCs constitute a heterogeneous group of cells. They were the first MSCs to be isolated and reported in 1966 by Friedenstein and colleagues. They isolated a population of cells from guinea-pig BM with fibroblast-like plastic adherent phenotype in vitro with colony-forming abilities. These cells could self-renew, express mesenchymal markers, such as CD29, CD44, CD73, CD90, and CD105, CD106, and CD166, and lacking in the expression of hematopoietic markers, such as CD14, CD34, and CD45, and capable of spontaneous bone formation in the diffusion chambers (in vitro), besides inducible osteogenesis in the presence of transitional epithelium (Friedenstein et al. 1970). Later on, in 1991, Caplan suggested changing the name to "mesenchymal stem cells" and ascribed these cells as responsible for bone and cartilage formation in the embryo and responsible for their repair and turnover in adult life (Caplan 1991). Caplan also described the role of some extrinsic factors and the inherent genomic potential to control the rate and phenotype of the cells in the emerging tissue from these cells and predicted their emergence as a novel therapeutic modality for the repair of skeletal tissues. While describing the mesengenic process, Caplan described a pivotal role of MSCs in terms of undergoing proliferation, lineage commitment, and terminal differentiation to adopt the desired phenotype, that is, osteogenic, chondrogenic, and adipogenic (Caplan 1994). Going further, in 2017, Caplan has now suggested to rename MSCs as "medicinal signaling cells" to reflect on the ability of MSCs to migrate to the site of injury and participate in the repair process (Caplan 2017).

Although BM-derived MSCs are the most extensively studied, well-characterized, and widely used cells in clinical settings, MSCs represent only 0.001–0.01% of mononuclear cells in the BM. Due to this small fraction, BM-derived MSCs necessitate in vitro expansion to acquire sufficient numbers for

in vivo use (Pittenger et al. 1999). In this regard, various in vitro strategies have been adopted that include culturing of cells under serum-free conditions to 3D-culturing that simulate the natural habitat of the cells (Bhat et al. 2021).

Advantages of BM-Derived MSCs

BM-derived MSCs show multipotent differentiation potential toward different embryonic lineages. These lineages include the mesodermal lineage, that is, adipocytes, chondrocytes, osteocytes, endothelial cells, and skeletal muscles, ectodermal lineage, that is, neurons, retinal cells, and endodermal lineage, that is, hepatocytes and insulin-producing cells (Friedenstein et al. 1970; Polisetti et al. 2010). However, the ability of MSCs to adopt different lineage phenotypes is considered tissue source of MSCs-dependent. For example, BM-derived MSCs show stronger osteogenic but lower adipogenic potential than Ad-MSCs, but they both offer similar chondrogenic potential (Xu et al. 2017). Similarly, a comparison of synovial membrane-derived MSCs and BM-derived MSCs showed that the latter have enhanced osteogenic differentiation potential, although their chondrogenic potential was similar (Gale et al. 2019). This multilineage differentiation enables BM-derived MSCs to provide vast therapeutic applications for different diseases in regenerative medicine (Haider and Ashraf 2005).

BM-derived MSCs are still the most used MSCs in clinical settings (Wang et al. 2016a). Interestingly, BM-derived MSCs were the first "stem cell drug" to be registered by the Food and Drug Administration (FDA) as a drug (Prochymal™) against graft vs. host rejection disease (GvHD) (Prasad et al. 2011). Presently, BM-derived MSCs have more than 400 registered clinical trials with almost 160 completed trials involving a broad scope of immune, chronic, cardiovascular, and degenerative diseases, for instance, diabetes mellitus, neurodegenerative diseases, and heart diseases (ClinicalTrials.gov 2019).

Limitations of BM-Derived MSCs

Despite the advantages mentioned earlier, a few limitations have led to cautious advancement of BM-derived MSCs to the clinic in regenerative medicine. For example, the isolation of BM-derived MSCs is very complicated and more invasive than UCB, adipose tissue, and WJ. Harvesting of BM-derived MSCs can cause possible discomfort and morbidity due to the invasive BM aspiration process (Mazini et al. 2019). In addition, BM-derived MSCs isolation may require centrifugation to separate mononuclear cells from RBCs, platelets, and granulocytes (Naji et al. 2019). Moreover, the limited yield of cells necessitates in vitro expansion, which may result in altering the immunogenic status and differentiation potential of cells. However, several reports have reported that BM-derived MSCs exhibit signs of early senescence during in vitro culture expansion. All these problems directed researchers to search for alternative sources of MSCs.

Adipose Tissue-Derived MSCs

Ad-MSCs constitute a heterogeneous population of cells isolated from adipose tissue of different body parts. They exhibit all the essential characteristics of the MSCs

proposed by the ISCT, namely plastic adherence, expression of mesenchymal cell-specific surface membrane markers and lacking in hematopoietic stem cell-specific surface membrane markers, and the ability to undergo trilineage differentiation, that is, adipocytes, chondrocytes, and osteocytes. Like the other tissues-derived MSCs, Ad-MSCs also offer several unique advantages over other sources of MSCs (Bourin et al. 2013; Fathi and Farahzadi 2009). Besides the typical mesenchymal surface markers, Ad-MSCs also express primitive cell surface markers of core circuity of self-renewal, that is, OCT4, NANOG, SOX2, and the neurogenic lineage-specific genes, that is, NEUROD1, PAX6, and SOX3 (Gentile et al. 2019). Although similar in surface marker expression to BM-derived MSCs, the yield of Ad-MSCs is approximately 500 times more than the BM per gram of the respective tissue (Hall et al. 2010). They show higher proliferative potential without losing much of their stemness properties (Zhu et al. 2008). Another study comparing BM-derived MSCs and Ad-MSCs derived from patients with coronary artery disease has shown the superiority of Ad-MSCs in their response to low oxygen culturing that renders them more suitable for transplantation for cardiovascular therapy (Adolfsson et al. 2020).

Advantages of Adipose Tissue-Derived MSCs

Ad-MSCs propose several advantages over BM-derived MSCs that make them of special interest in the clinical perspective. Firstly, the ease of availability in tissue harvesting and isolation of the Ad-MSCs from the harvested tissue constitutes a significant advantage of these cells. Similarly, Ad-MSCs can be isolated from subcutaneous fats of different parts of the body, including the abdomen, thighs, and buttocks, with far less invasive procedure than the BM-derived MSCs. This less invasive procedure is associated with lesser donor morbidity, pain, or complications than the BM-MSCs (De Ugarte et al. 2003). Second, the lipoaspirate showed a higher population of MSCs, reaching 10% compared to the scarce population of 0.01–0.001% of the mononuclear cells of BM-derived MSCs (Kern et al. 2006; Zhu et al. 2008). Ad-MSCs from lipoaspirate might reach 500 times that obtained from BM (Marigo and Dazzi 2011). Thirdly, and most importantly, Ad-MSCs showed better-culturing abilities, greater proliferation capacity, and they can maintain their phenotype longer in culture (Kunze et al. 2020). These properties are very significant in producing many "clinical grade" MSCs for clinical applications. Besides, several reports have shown that Ad-MSCs could differentiate into different cell types of the three developmental germ layers (endoderm, mesoderm, and ectoderm), including adipocytes, osteoblasts, chondrocytes, neurocytes, and hepatocyte (Chen et al. 2016; Dai et al. 2016). These properties render Ad-MSCs as a front-runner for clinical applications.

Limitations of Adipose Tissue-Derived MSCs

Although Ad-MSCs clinical applications have increased lately, several limitations must be overcome (Mazini et al. 2020). Ad-MSCs yield, culture properties, and differentiation capacities are dependent on several factors, including the harvesting/isolation methods and the donor health conditions (Zhang et al. 2020a). Harvesting cells by excision has a better yield than lipoaspiration and directs the cells toward mesoderm and ectoderm differentiation rather than the endoderm associated with the

lipoaspirate (Bian et al. 2016; Gnanasegaran et al. 2014). Besides, no single controlled procedure exists to isolate reproducible Ad-MSCs following Good Manufacturing Practice (GMP) (Kassem and Kamal 2020c; Kim et al. 2015; Koh et al. 2016).

Moreover, Ad-MSCs properties are highly affected by the medical comorbidity of the patients. For example, obesity of the donor induces early senescence in the Ad-MSCs. The cells obtained from obese patients showed inadequate proliferative capacity due to upregulation of p16, p53, IL-6, and MCP1, but their emigrational capability was sustained (Conley et al. 2020). Similarly, results about the declining function of adipose tissue-derived stem cells have also been reported with extensive passaging (Zhu et al. 2008). It has also been reported that the differentiation capacity and the proliferative potential of Ad-MSCs are highly influenced by the donors' conditions such as age, BMI, suffering from diabetes mellitus, exposure to radiotherapy, or endocrine therapy (Varghese et al. 2017). For example, the differentiation potential of Ad-MSCs from diabetic patients is significantly lost due to generalized inflammatory response in the patients (Barbagallo et al. 2017). However, further investigations are required to confirm the clinical significance of such differences (Zhang et al. 2020a).

Isolation of Adipose Tissue-Derived MSCs

Generally, adipose tissue is harvested using different methods, that is, liposuction, resection, micro-fat harvesting, etc., and each one of the methods has advantages and limitations and significantly affect the yield and characteristics of the Ad-MSCs thus derived therefrom, as discussed elsewhere (Prantl et al. 2021). The tissue harvesting step is followed by subsequent washing of the harvested adipose tissue to remove the contaminating erythrocytes. The tissue-digestion step is next followed in a typical protocol that may be achieved either mechanically or enzymatically and centrifuged to separate the stromal vascular fraction (SVF). The cells of SVF are then suspended in serum-containing media for culture and expansion of Ad-MSCs.

Coleman's method is most commonly used to harvest adipose tissue from among the many methods reported in the literature to date for harvesting adipose tissue (Alstrup et al. 2020). The method of harvesting can influence the number of Ad-MSCs obtained, the stemness, the proliferative capacity, the multilineage potency, and the aging rate of the isolated cells (Bajek et al. 2017). For example, it was reported that surgical resection is associated with a large number of Ad-MSCs showing a higher rate of clonogenicity. On the other hand, cells isolated by lipoaspiration showed increased proliferation and the least aging rate. Some other methods for the isolation of Ad-MSCs, the process of adipose tissue harvesting may involve surgical resection, power-assisted lipoaspiration, laser-assisted lipoaspiration, etc. (Khazaei et al. 2021; Fontes et al. 2018).

As for the digestion of the adipose tissue, mechanical digestion methods, such as shaking, centrifugation, or filtration, are cost-effective but these are time-intensive and harsh on the cells to inflict mechanical damage on the cells. This may also lead to a poor yield of the viable cells (Khazaei et al. 2021). Several enzymes were used for digestion of the adipose tissue, such as collagenase, trypsin, and dispase (Bourin et al.

2013; Yang et al. 2011; Zhu et al. 2013), However, adipose tissue digestion with collagenase is the gold standard among the currently used methods (Banyard et al. 2015). However, as collagenase is of bacterial origin, using high-grade enzyme preparation and removing excess enzyme by centrifugation is essential to ensure safety and decrease toxic effects on the cells for subsequent clinical applications (Aguena et al. 2012). Still, the methods for adipose tissue harvesting and isolation of Ad-MSCs from the harvested tissue require more efforts to standardize and apply GMP to ensure the production of "clinical grade" cells for patient applications.

Clinical Applications of Adipose Tissue-Derived MSCs

Several excellent reviews have been published discussing the clinical applications of Ad-MSCs for different diseases (Shukla et al. 2020; Zhang et al. 2020a). Zhang and colleagues have presented an excellent review of various applications of Ad-MSCs for wound healing and the treatment of bone, cartilage, nervous system, liver, heart, skin, and even trachea and gall bladder. These studies are either preclinical or clinical studies. Shukla and co-workers (2020) published a review to discuss extracellular vesicles with a focus on exosomes derived from Ad-MSCs for clinical applications. These reviews reflect an increasing interest of the researchers and the ever-growing applications of Ad-MSCs in regenerative medicine to treat various diseases.

Umbilical Cord-Derived MSCs

Human Umbilical Cord Structure and Function

During pregnancy, the placenta and growing fetus are connected by the UC, also called "the naval string" (Spurway et al. 2012) that prevents umbilical vessels from kinking, compression, or torsion during movement of the fetus, thus ensuring proper blood supply to the fetus (Kim et al. 2013). Anatomically, the human UC comprises an outer layer of amniotic epithelium enclosing a vein and two arteries embedded within a mucoid connective tissue. The mucoid connective tissue enclosing the three umbilical vessels or the UC matrix is known as "Wharton's jelly." Thomas Wharton first described WJ in 1656 (Wharton 1656). In the early 1970s, UCB was reported as a natural reservoir and a rich source of hematopoietic stem cells (Knudtzon 1974). Nowadays, UCB has many therapeutic applications for various hematopoietic and non-hematopoietic disorders in the clinics (Munoz et al. 2014; Roura et al. 2015). Given their extensive regenerative potential, the collection, and banking of UCB and its derivative stem cells as part of personalized medicine (Harris 2014), including very small embryonic-like stem cells (Bhartiya et al. 2012), is gaining popularity for use as an autologous source of cells for cell-based therapy (Badowski and Harris 2012; Um et al. 2020).

The first report providing robust evidence that WJ-derived stromal cells can be classified as MSCs was published by Wang et al. (2004), which showed that the cells derived from WJ displayed a fibroblast-like phenotype when expanded in vitro (Wang et al. 2004). Also, these cells expressed high levels of MSCs markers

CD29, CD44, CD73, and CD105 but lacked the expression of the hematopoietic-specific markers, that is, CD31, CD34, and CD45. Moreover, those cells could differentiate into osteogenic and adipogenic lineages under favorable culture conditions (Wang et al. 2004). More interestingly, treatment with 5-azacytidine or cardiomyocyte-conditioned medium, the cells expressed cardiomyocyte-specific markers, that is, N-cadherin, cardiac troponin-I. UCB is also a source of MSCs, but to a much lesser extent than WJ (Arutyunyan et al. 2016; Sibov et al. 2012). Compared with the success rate of harvesting MSCs, it has to be almost 100% from WJ compared to a meager 6% from UCB (Shetty et al. 2010). A direct comparison of various protocols of MSCs isolation from UCB and WJ tissue has been provided by Salehinejad et al. (2012). These protocols are developed to optimize the methodological aspects of the procedure. The latest advancement in this regard is the proteolytic enzyme-free method for the isolation of MSCs (Singh et al. 2019). It is noteworthy that the therapeutic potential of MSCs derived from these two UC compartments could differ significantly in preclinical and clinical settings (El-Demerdash et al. 2015; Kassem and Kamal 2020b).

Advantages of WJ-Derived MSCs

WJ-derived MSCs possess exceptional stemness properties due to the presence of a primitive cell population (Troyer and Weiss 2008; Fong et al. 2011). WJ-derived MSCs have several advantages over other types of stem cells. First of all, they can be easily isolated from the readily available UC tissue discarded at labor as medical waste. Thus, unlike BM-derived MSCs, the isolation of WJ-derived MSCs is non-invasive, and unlike ESCs, the availability and use of WJ-derived MSCs are without any moral and ethical concerns (Hass et al. 2011). Second, WJ-derived MSCs isolated from neonatal tissue have more primitive characteristics than adult tissue-derived MSCs (Frausin et al. 2015). They are believed to represent an intermediate state between ESCs and adult stem cells (Marino et al. 2019). Interestingly, WJ-derived MSCs exhibit a mix of human ESCs and MSCs-specific markers and maintain stemness for several serial passages (Nekanti et al. 2009). Interestingly, while they possess several characteristics of ESCs, they do not form teratomas upon transplantation (Troyer and Weiss 2008). Third, WJ-derived MSCs are immune-privileged due to human leukocyte antigen-G (HLA-G) expression, besides lacking in the expression of human leukocyte antigen- antigen D-related (HLA-DR) like other MSCs types (La Rocca et al. 2009). These immunomodulatory properties were reported to prevent rejections even after xeno-transplantation of post-differentiated MSCs without immunosuppression (Moffett and Loke 2003). A direct comparison of MSCs derived from four different tissues, adipose tissue, BM, WJ, and UCB, have shown that WJ-derived MSCs possess the highest immunomodulatory effects (Li et al. 2014). Given their excellent immunomodulatory properties, WJ-derived MSCs are an attractive choice when immune cellular therapy is required (Najar et al. 2012). Finally, WJ-derived MSCs, and their counterparts isolated from UCB, both have an excellent potential for biobanking (Chatzistamatiou et al. 2014).

Limitations of WJ-Derived MSCs

Despite many advantages of WJ-derived MSCs over other stem cell types, several limitations have hampered their successful translation from the bench-to-bedside. For example, among these limitations is the requirement of in vitro expansion, which is significantly affected by culture conditions. For WJ-derived MSCs, the serum is an essential component of the culture as well as cryopreservation media; however, the use of culture-expanded cells causes serious immunological reactions due to the residual animal serum proteins from animal serum slowly settled on the cells' surface during long-term culture. This adversely alters the immunological characteristics of the cells besides reducing their therapeutic benefits post engraftment (Li et al. 2015b).

It is noteworthy that several alternatives of xenogeneic serum have been suggested to achieve humanized stem cell culture using human alternatives for the xenogenic protein (Bieback et al. 2009; Tekkatte et al. 2011). Given its unique growth factor and cytokine-rich composition, one proposed alternative is platelet-rich plasma (PRP) (Kandoi et al. 2018; Haider 2017). The authors observed at least a twofold increase in the yield from the explant culture with significantly preserved immunomodulatory properties of the cells. Besides enhancing the safety aspects of the in vitro cultured cells, xeno-free culture expansion also improves their therapeutic functionality (Kang et al. 2020). Besides the xenoprotein-free culture system, Obradovic et al. have reported in vitro expansion protocol that closely mimics the natural habitat of the cells in terms of low oxygen presence (Obradovic et al. 2019). The authors reported that the cells cultured under 3% oxygen conditions retained their expression of OCT4A, OCT4B, NANOG, and SOX2 expression and showed excellent migratory capacity. Another limitation of WJ-derived MSCs is the lack of standardized protocols for their processing, cryopreservation, or banking. These include the isolation of WJ-derived MSCs from fresh tissue versus frozen ones (Fong et al. 2016).

Additionally, during prolonged in vitro culture, WJ-derived MSCs have been reported to show changes in their transcriptome profile, compared to the early passage cells, where these cells might exhibit a progressive decline in their physiological properties; a phenomenon known as "Cellular aging" (Gatta et al. 2013). Regarding the clinical application of WJ-MSCs, similar to stem cells from other tissue sources, various parameters, such as the best route of administration, time of injection, dose of the cells, frequency of doses, as well as the time intervals between multiple injections, remain controversial issues (Kamal and Kassem 2020).

Isolation Methods of WJ-Derived MSCs

Various isolation protocols have been optimized and reported to generate WJ-derived MSCs from the UC tissue. Primarily, these protocols are based on tissue explant and chemical enzymatic digestion approaches, which are incidentally the two methods for WJ-derived MSCs isolation (Han et al. 2013; Xu et al. 2010). Sometimes a mix between enzymatic and mechanical digestion approach, using enzymes like collagenase with/without hyaluronidase or trypsin, followed

by passing the treated WJ through an 18-G needle syringe (Azandeh et al. 2012). The explant method is based on the ability of the MSCs to move and migrate from the tissue and adhere to the plastic surface (Goyal et al. 2018). It is generally described as cutting the UC into shorter pieces, excision of the blood vessels, and fine chopping of the WJ sections of the cord tissue, followed by plating those fine fragments with complete media. Besides being cost-effective and reproducible, the primary advantage of the tissue explant method is that it does not affect the vitality of cells. It is noteworthy that enzymes may degrade the cell membrane during enzymatic digestion, resulting in further damage to the cells (La Rocca et al. 2009). Hua and co-workers concluded that the 10 mm size tissue explant method is the optimal protocol for isolating MSCs from UC (Hua et al. 2013).

Clinical Applications of WJ-Derived MSCs

Over the last many years, the published data from the preclinical experimental studies have demonstrated the safety and highly promising therapeutic potential of WJ-derived MSCs. In vitro characterization reveals a primitive cell population positive for OCT4 and NANOG expressing cells (Pirjali et al. 2013; Shaer et al. 2014). Based on the hypothesis that tissue source may determine the biological characteristics and functionality of the derivation MSCs, a recent study by Laroye and colleagues have shown a better profile of WJ-derived MSCs as compared to the BM-derived MSCs in mice model of sepsis by caecal ligation and puncture (Laroye et al. 2019). Various clinical trials have been conducted to test the safety, feasibility, and efficacy of WJ-derived MSCs-based cell therapy for many diseases, including graft versus host disease (GvHD), bone/cartilage disease, immunological diseases, diabetes mellitus and its complications, cardiac disease, liver disease, neurological disorders, as well as cancer (Abbaszadeh et al. 2020). Many of these clinical trials have been completed and demonstrated the safety and efficacy of WJ-MSCs (Can et al. 2017). The latest application is the effective use of WJ-derived MSCs in patients suffering from COVID-19 pneumonia (Zhang et al. 2020b). Intravenous infusion of WJ-derived MSCs has shown promising results due to their strong immunomodulatory actions (Shi et al. 2021).

Therapeutic Strategies of Using MSCs

MSCs have made their mark as a promising therapeutic option for many regenerative medicine applications. Originally, MSCs were thought to mediate their reparative potential by virtue of a multilineage differentiation capability that enabled them to replace damaged cells (Mahmood et al. 2003; Murphy et al. 2003). However, later on, a growing body of evidence has revealed that in response to tissue injury, MSCs home into the site of tissue damage and can mediate tissue repair via paracrine action through the release of soluble and insoluble secretome and immunomodulatory activities (Chen et al. 2008; Karp and Teo 2009). The various therapeutic strategies of MSCs have been summarized in Fig. 2.

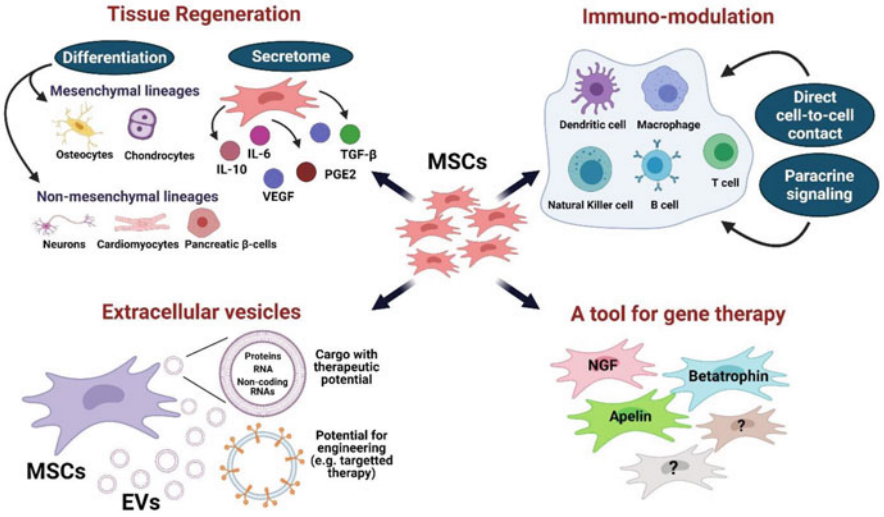

Fig. 2 Therapeutic strategies of MSCs including their ability to regenerate damaged tissue, either by direct mechanisms or indirectly via secretion of cytokines, growth factors, exosomes, and immune system modulating agents. (Created with Biorender.com)

Tissue Regeneration

Irrespective of their source, MSCs have demonstrated remarkable capability to regenerate various tissue types, especially those of mesodermal origin, such as muscle, bones, and cartilage (Pittenger et al. 2019; Le et al. 2020), as well as cardiomyocytes, neurons, and pancreatic β-cells (Farini et al. 2014).

For instance, MSCs have exhibited an excellent regenerative capacity for bones and cartilage repair, both in experimental as well as clinical settings (Kangari et al. 2020). Elucidating the cascade of events involved in the bone repair process, the transplanted cells caused the mobilization of macrophages, induced functional switch from pro-inflammatory to pro-resolving phenotype, and recruitment of endothelial progenitor cells and cells with osteogenic potential intrinsically from the BM (Tasso et al. 2013). Tadoeschi et al. proposed that transplanted MSCs modified the in vivo environment conducive to angiogenesis and bone regeneration (Todeschi et al. 2015). Additionally, the transplanted cells also undergo endothelial differentiation to participate in the formation of the new capillary network (Chen et al. 2009). Various strategies have been adopted to enhance the efficiency of bone repair. For example, Zhou et al. used in vitro osteogenically induced human MSCs for bone tissue engineering in mice to repair skull defects (Zhou et al. 2015). The latest advancement in this regard is the use of scaffold-based delivery of MSCs to enhance their efficacy. Li et al. used a composite scaffold of WJ and chondroitin sulfate loaded with human UC-derived MSCs knee repair in a rodent model (Li et al. 2021). At a molecular level, a variety of mediators with regenerative effects include,

transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), indoleamine 2,3-dioxygenase (IDO), bone morphogenic proteins (BMPs), and others (Granero-Moltó et al. 2009; Qin et al. 2014) have a significant role therein.

Interestingly, MSCs-based therapy also represents a novel efficient therapeutic strategy for cartilage repair owing to its outstanding chondrogenic differentiation potential (Le et al. 2020). MSCs secrete several bioactive factors, which help to restore the extracellular matrices (ECMs) that are essential for the recovery of cartilage functions (Lories and Luyten 2011). Finally, engineered scaffolds have also been used in combination with MSCs and exogenous biochemical stimuli, and demonstrated profound progress in cartilage regeneration (Smith and Grande 2015).

Immunomodulation of MSCs

Although MSCs were initially used to exploit their regenerative capacity and reparability, however, the therapeutic potentials of MSCs have been attributed more to paracrine action and immunomodulation with partial contribution from multilineage differentiation (Ceccarelli et al. 2020). It is now well-established that MSCs exhibit a hypoimmunogenic phenotype as they lack the major histocompatibility class II (MHC-II) and release of immunomodulatory cytokines (Lacy et al. 2021). Thus, these characteristics enable them to evade recognition by the recipient immune system (McIntosh et al. 2006; Puissant et al. 2005). These immunemodulatory effects of MSCs collectively involve cell-to-cell contact-dependent mechanisms and paracrine effects by releasing a plethora of cytokines and growth factors that regulate the immune functions besides decreasing the secretion of pro-inflammatory cytokines (Sotiropoulou et al. 2006; Zhou et al. 2018).

Interestingly, Ad-MSCs are superior in immune-modulatory effects than BM-derived MSCs in a matched donor (Melief et al. 2013). This superiority can be attributed to Ad-MSCs' ability to suppress lymphocyte proliferation, inhibit monocytes-derived dendritic cells, natural killer (NK) cytotoxic activities, and the secretion of higher levels of cytokines (Melief et al. 2013; Russell et al. 2016; Valencia et al. 2016).

MSCs influence innate immunity and adaptive immunity through the two axes mentioned earlier, the cell-to-cell contact and the paracrine action. MSCs affect almost all the immune cells through cell-to-cell contact, including T-cells, B-cells, NK cells, macrophages, and DC (Zhou et al. 2019). It has been documented that MSCs inhibit naïve T-cells and memory T-cell responses to communicate with the antigen-presenting cells (Krampera et al. 2003) and inhibit the proliferation of CD4+ and CD8+ T-cells through galectin-1 (Gieseke et al. 2010). Besides their effects on T-cells, MSCs also increase the survival of quiescent B-cells through cell-to-cell contact (Franquesa et al. 2015).

It is important to mention that infused MSCs, such as UC-MSCs, reside in the lungs, are phagocytosed by the monocytes causing changes in the monocyte's phenotype and function and modulate the adaptive immune system responses

(de Witte et al. 2018). Moreover, MSCs suppress the cytotoxic NK cells' granular polarization (Hu et al. 2019). In this regard, Ad-MSCs are known to switch activated inflammatory M1 macrophages to an M2 macrophage-like phenotype via prostaglandin E2 (PGE2) release (Manferdini et al. 2017).

Regarding the second axis of cytokine secretion via paracrine mechanisms, the MSCs secretome consists of many cytokines, chemokines, and growth factors, such as transforming growth factor-β1 (TGF-β1), PGE2, IFN-γ, hepatocyte growth factor (HGF), indoleamine-pyrrole 2,3-dioxygenase (IDO), tumor necrosis factor-α (TNF-α), fibroblast growth factor (FGF), and nitric oxide, besides many others (Salgado et al. 2010; Li and Hua 2017; Zhou et al. 2019). Interestingly, these bioactive molecules are encapsulated in extracellular vesicles, such as exosomes, micro-vesicles (MVs), or apoptotic bodies, depending on the type of cells of their origin (Ferreira et al. 2018).

MSCs inhibit T-helper 17 (Th17) differentiation by IL-10, PGE2 induction, and IL-17, IL-22, and IFN-γ inhibition (Ghannam et al. 2010). Moreover, MSCs can also inhibit CD4+ T-cells activation via the secretion of programmed death ligand-1 (PD-L1) and PD-L-2 (Davies et al. 2017). MSCs can also inhibit IL-2 induced NK cells activation by the secretion of IDO and PGE2 (Spaggiari et al. 2008). Additionally, MSCs-derived IL-6 protects neutrophils from apoptosis and preserves them in the BM niche (Raffaghello et al. 2008). IL-6 can also prevent the differentiation of monocytes toward an anti-inflammatory IL-10-producing phenotype (Melief et al. 2013). Also, MSCs-derived PGE2 empower MSCs to suppress the differentiation of monocytes to mature DCs (Spaggiari et al. 2009).

Even the conditioned media (CM) of MSCs containing both soluble and insoluble factors (MSCs-derived exosomes) shows immune-modulatory effects emphasizing the involvement of paracrine actions of the cells. MSC-derived exosomes have been shown to augment neutrophil viability. In contrast, MSCs-derived CM increases neutrophil function, demonstrating that both MSCs-derived exosomes and CM are helpful for improving immunity by modulating neutrophils (Mahmoudi et al. 2019). Additionally, MSCs-derived EVs can attenuate DC maturation, as well as pro-inflammatory cytokines (IL-6 and IL-12p70) and upregulation of the anti-inflammatory cytokine TGF-β (Reis et al. 2018). Besides, MSCs-derived exosomes trigger macrophage polarization (Lo Sicco et al. 2017) by enhancing the formation of anti-inflammatory M2 macrophages over M1-like inflammatory macrophages via downregulation of IL-23 and IL-22 (Hyvarinen et al. 2018).

Extracellular Vesicles/Exosomes Derived from MSCs

Interestingly, a growing body of research has revealed that MSCs mediate several therapeutic/beneficial effects via secreting extracellular-vesicles (EVs) (Newton et al. 2017). Generally, the secreted membrane-enclosed vesicles, collectively called EVs, include exosomes, ectosomes, microvesicles, apoptotic bodies, and other EV subsets (Lötvall et al. 2014).

Initially considered the "garbage bags for the disposal of cellular waste," MSCs-derived exosomes are now emerging as important mediators of intercellular communication. They have a specific cargo of DNA, mRNA, miRNA, proteins, growth factors, etc. (Haider and Aramini 2020). The content profile of exosomes is cell-type dependent and constantly fluctuates with many determinant factors, including the microenvironmental factors in which the cells are present at a particular time point. They have notably exhibited therapeutic potential for various diseases and are fast emerging as innovative tools in nanomedicine (Kassem and Kamal 2020a; Norouzi-Barough et al. 2021; Wei et al. 2021). Based on these characteristics, exosomes are defined as nano-sized bioactive vesicles derived from the cell's endosomal membrane system and secreted into surrounding body fluids (Théry et al. 2018).

Exosomes can reprogram the recipient cells acting as "signalosomes" for controlling fundamental cellular functions by transferring their cargo to the recipient cells (Gangoda et al. 2015; Bjorge et al. 2018). Recently, exosomes have sparked great interest as a potential cell-free therapy alternative to the current cellular therapies (Haider and Aslam 2018). MSCs-derived exosomes are gaining special attention among the different cell types due to their unique cargo and ease of manipulation (Janockova et al. 2021). Like their parent cells, exosomes stimulate functional recovery and cellular regeneration in various disease conditions (Derkus et al. 2017). However, unlike their parent cells, they have no critical safety concerns; they pose no risk of tumor formation or any concern regarding the immune rejection by the recipient, and they can pass through biological barriers, that is, the blood-brain barrier, more efficiently. Moreover, since exosomes are naturally equipped to mediate intercellular communication via the transfer of genetic information to recipient cells, they can be utilized as drug delivery systems for gene therapy applications (Colao et al. 2018). MSCs from various tissue sources, Ad-MSCs, BM-derived MSCs, and UCB-derived MSCs, are efficient and established mass producers of exosomes (Yeo et al. 2013; Janockova et al. 2021). Furthermore, MSCs-derived exosomes have been suggested to act through the protein-based mechanism of action as well as micro-RNAs and/or packaged metabolites (Luther et al. 2018; Showalter et al. 2019; Toh et al. 2018).

Conclusively, exosomes derived from MSCs provide a novel platform for a wide array of therapeutic strategies for various disease conditions and eventually may develop into a standardized allogeneic off-the-shelf immunomodulatory and regenerative therapeutics. After extensive evaluation for safety and efficacy studies in the preclinical experimental animal models, they have moved to clinical assessment and www.clinicaltrials.gov shows 226 registered clinical studies involving evaluation of exosome delivery for various diseases, including cardiovascular diseases, stroke, cancer, and COVID-19 pneumonia.

MSCs Act as an Efficient Tool for Gene Therapy

Over the past few years, due to their homing capacity (Karp and Teo 2009) and immunomodulatory activities (Song et al. 2020), MSCs have been nominated as an

efficient tool for gene therapy to treat several pathological conditions, lying at the intersection of cell and gene therapy. Several techniques have been used to generate genetically modified MSCs leading to the up-regulation or down-regulation of native genes related to their regenerative potential or immunomodulatory functions. Alternatively, genetic engineering has been used to modulate them to serve as carriers of transgenes by introducing foreign genes of interest. The genetically modulated cells serve as tiny little factories that continue to secrete the product of the overexpressing genes that significantly contribute to the therapeutic effects of the genetically modulated MSCs (Varkouhi et al. 2020). Such a novel approach using genetically-modified MSCs as gene delivery vehicles widely expand the spectrum of the possible applications of MSCs in various diseases (Oggu et al. 2017).

Interestingly, several cell-therapy pre-clinical studies employing genetically modified MSCs in various critical illness conditions such as acute myocardial infarction, acute liver failure, or even acute lung injury revealed promising results (Varkouhi et al. 2020). Furthermore, genetically engineered MSCs have been used as a vehicle to deliver biological agents to various tumors in preclinical cancer models. In these studies, MSCs have been engineered to express prodrug-converting enzymes, cytokines, pro-apoptotic factors, or antiangiogenic agents either single or multiple transgene overexpression to promote their therapeutic benefits after transplantation (Jiang et al. 2006; Mohr and Zwacka 2018; Mosallaei et al. 2020). Simultaneous overexpression of Akt and angiopoietin-1 has been shown to enhance their endothelial commitment (Lai et al. 2012). MSCs have also been used as carriers of microRNAs, either by genetic modulation to improve their survival and reparability post engraftment (Kim et al. 2012). Transgene overexpression in MSCs has also been used for their reprogramming to pluripotency (Buccini et al. 2012). Conclusively, MSCs indeed represent an efficient tool for gene-therapy applications in regenerative medicine.

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Considerations for Clinical Use of Mesenchymal Stromal Cells

3

Abdelkrim Hmadcha, Bernat Soria, Juan R. Tejedo, Francico J. Bedoya, Jose Miguel Sempere-Ortells, and Tarik Smani

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A. Hmadcha (✉)
Department of Biotechnology, University of Alicante, Alicante, Spain

University of Pablo de Olavide, Sevilla, Spain

Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid, Spain
e-mail: khmadcha@upo.es

B. Soria
University of Pablo de Olavide, Sevilla, Spain

Department of Physiology, Institute of Bioengineering-Alicante Institute for Health and Biomedical Research (ISABIAL), University Miguel Hernández School of Medicine, Alicante, Spain
e-mail: bernat.soria@umh.es

J. R. Tejedo · F. J. Bedoya
Department of Molecular Biology and Biochemical Engineering, University of Pablo de Olavide, Sevilla, Spain

Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid, Spain
e-mail: jrtejhua@upo.es; fbedber@upo.es

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Abstract

The clinical application of stem cells continues to fascinate the scientific and clinical communities. Despite the controversies surrounding this field, it is clear that stem cells have revolutionized regenerative medicine. Cell therapy is a progressively growing field that is moving fast from preclinical model development to clinical application. In this regard, outcomes obtained from clinical trials reveal the therapeutic potential of stem cell-based therapy that deals with unmet medical treatment for several disorders with no therapeutic alternatives. The application of stem cells in regenerative medicine is addressing a wide range of clinical conditions using various types of stem cells. Mesenchymal stromal cells (MSCs) have been established as promising candidate sources of universal donor cells for cell therapy due to their contributions to tissue and organ homeostasis, repair, and support by self-renewal and multi-differentiation, as well as by their anti-inflammatory, anti-proliferative, immunomodulatory, trophic, and pro-angiogenic properties. Various diseases have been successfully treated by MSCs in animal models. Additionally, hundreds of clinical trials related to the potential benefits of MSCs are in progress or have concluded satisfactorily. However, although all MSCs are considered suitable to exert these functions, dissimilarities have been found among MSCs derived from different tissues. The same levels of efficacy and desired outcomes have not always been achieved in the diverse studies that have been performed thus far. Therefore, collecting information regarding the characteristics of MSCs obtained from different sources and the influence of other medical and physiological conditions on MSCs is important for

J. M. Sempere-Ortells
Department of Biotechnology, Immunology Division, University of Alicante, Alicante, Spain
e-mail: josemiguel@ua.es

T. Smani
Cardiovascular Pathophysiology, Institute of Biomedicine of Seville (IBiS), University Hospital of Virgen del Rocío/University of Seville/CSIC, Seville, Spain

Department of Medical Physiology and Biophysics, University of Seville, Seville, Spain

Biomedical Research Networking Centers of Cardiovascular Diseases (CIBERCV), Madrid, Spain
e-mail: tasmani@us.es

assuring the feasibility, safety, and efficacy of cell-based therapies. This chapter will update and discuss the state of the art in MSCs' cell-based therapies and provide relevant information regarding factors to consider for the clinical application of MSCs.

Keywords

Advanced therapy · Cell therapy · Clinical trial · Good manufacturing practice · Immunomodulation · Inflammation · Medicinal products · Mesenchymal stromal cells · Trophic factors

Abbreviations

Ad-MSCs	Adipose tissue-derived MSCs
AGEs	Advanced glycation end products
ATMPs	Advanced therapy medicinal products
bFGF	Basic fibroblast growth factor
BM-MSCs	Bone marrow-derived MSCs
CAFs	Cancer-associated fibroblasts
Cas9	CRISPR-associated protein 9
CCL	Chemokine (C-C motif) ligand
CFU-F	Colony-forming unit fibroblast
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL12	C-X-C motif chemokine 12 (or SDF1)
Dkk-1	Dickkopf-1
ECs	Endothelial cells
EMA	European Medicines Agency
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GPS	Glycotransferase-programmed stereo substitution
GVHD	Graft-versus-host diseases
GVL	Graft-versus-leukemia
HCELL	Hematopoietic cell E-selectin/L-selectin ligand
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen-DR isotype
HLA-G5	Human leukocyte antigen-G5
HSCT	Hematopoietic stem cell transplantation
IBMIR	Instant blood-mediated inflammatory reaction
ICAM-2	Intercellular adhesion molecule 2
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor 1

IL	Interleukin
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
iPSCs	Induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
ITP	Immune thrombocytopenic purpura
LFA-3	Lymphocyte function-associated antigen 3 (or CD58)
MCP-1	Monocyte chemoattractant protein 1
MHC-HLA	Major histocompatibility complex-human leukocyte antigen
MMP-2	Matrix metalloproteinase 2
MSCs	Mesenchymal stromal cells
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PD-MSCs	Placenta-derived MSCs
PGE-2	Prostaglandin-E2
RA	Rheumatoid arthritis
SDF-1	Stromal cell-derived factor 1
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
STC1	Stanniocalcin-1
TALENs	Transcription activator nucleases
TbRIII	Type III TGF-β receptor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
TRAIL	TNF-related apoptosis-inducing ligand
Trx1	Thioredoxin-1
TSG-6	Tumor necrosis factor-stimulated gene-6
UCB-MSCs	Umbilical cord-derived MSCs
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
ZFNs	Zinc finger nucleases

Introduction

Regenerative medicine is a novel emerging medical approach that drives the current understanding of biological and medical processes and suggests new treatments. As defined by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA), advanced therapies include cell and gene therapy and tissue engineering (Iglesias-López et al. 2019). Advanced therapies open up a broad set of translational fields and targets in areas of unmet medical need. In this regard, cell-based therapy through the application of cells, either alone or engineered, as a pharmacologically active substance seeks to restore the functioning of damaged

tissues or organs through the protection of cellular integrity, the replacement of damaged cells, and the promotion of trophic, anti-inflammatory, and immunomodulatory effects among others. However, while the progression of cell-based therapy in early-phase clinical trials with patients has progressed promisingly, the translation from laboratory to bedside to late-phase clinical trials has not been as rapid as expected. It is necessary to consider that these new therapeutic alternatives also involve unknown side effects that must be detected and characterized in-depth to improve and ensure safety, feasibility, and efficacy of cell application (García-Bernal et al. 2021; Hmadcha et al. 2020; Soria-Juan et al. 2019; Escacena et al. 2015; Gálvez et al. 2013).

In this regard, mesenchymal stromal cells (MSCs) are the most common cell type used in cell-based therapy due to their unique biological properties, including easy expansion and culture. The MSC-Committee of the International Society for Cellular Therapy (ISCT-MSC) first proposed that plastic-adherent cells of the bone marrow (BM) generally described as "mesenchymal stem cells" should be defined as "multipotent mesenchymal stromal cells." In contrast, the term "mesenchymal stem cells" should be reticent for a subset of these cells that show stem cell activity by clearly stated criteria. As the acronym MSCs may be used to define both cell populations, the combined definition "mesenchymal stem/stromal cells" is probably more appropriate, especially when the "stemness" of the whole MSC population is not demonstrated (Horwitz et al. 2005). Recently, this committee offers a position statement to clarify the nomenclature of "mesenchymal stem/stromal cells." The ISCT-MSC committee continues to support the use of the acronym "mesenchymal stromal cells" but recommends that this should be complemented by the tissue origin from which the cells were derived, which would highlight tissue-specific properties: that they be referred to as "stromal" unless there are rigorous in vitro and in vivo evidence of their stemness supplemented by a robust matrix of functional assays to demonstrate the "mesenchymal stromal cells" properties. Thus, they should not be defined generically, but based on the intended therapeutic mode of action (Viswanathan et al. 2019).

The MSCs are now considered as "cellular medicament" but are widely accepted to represent a heterogeneous population of multipotent non-hematopoietic progenitor cells with varying degrees of stemness, which mean that they have self-renewal and multi-differentiation abilities, the capability to differentiate into multiple cell types, including adipocytes, chondrocytes, and osteoblasts, depending on in vitro culture conditions (Soria-Juan et al. 2019).

The MSCs reside in almost all tissues, are found in virtually all post-natal organs and tissues, and are derived from the mesodermal germ layer. Furthermore, MSCs can be obtained from easily accessible sources by minimally invasive methods (e.g., peripheral blood, adipose tissue) and can be rapidly expanded in large scale for clinical use (Escacena et al. 2015). This allows producing a patient-specific cellular medicament (e.g., autologous medicinal product) within a therapeutic time window. In addition, the possibility of obtaining MSCs from adult tissue circumvents the ethical issues associated with the use of embryonic source (Lo and Parham 2009;

Ramos-Zúñiga et al. 2012). MSCs are inexpensively isolated and are easily expanded in vitro due to their fibroblastic characteristic and high adherence to plastic. MSCs are characterized by a specific pattern of membrane markers, consisting of the expression of CD73, D90, and CD105 and the absence of expression of CD14, CD34, CD45, and human leukocyte antigen-DR (HLA-DR), making them promising candidate sources of donor cells for use in cell-based therapy and transplantation (Horwitz et al. 2005).

MSCs function in tissue repair and support, contributing to tissue homeostasis. Even though the exact origin of MSCs remains elusive, there is strong evidence that MSC progenitors are found in the perivascular zone (Escacena et al. 2015) in an environment that promotes a quiescent state, ensuring the maintenance of homeostasis. Upon tissue damage, MSCs enter the bloodstream and are attracted to pro-inflammatory cytokines in the areas of injury. Therefore, MSCs have been termed "guardians of inflammation" (Prockop and Oh 2012). The cytoskeleton, extracellular matrix molecules, cell-cell contacts, adhesion ligands, and receptors are involved in the repair process. While the exact mechanisms related to MSCs' migration to specific sites and through the endothelial cell layer are still unknown, chemokines and their receptors may play a role in this process (Hmadcha et al. 2020; Petrie et al. 2009).

Furthermore, MSCs' survival, permanent engraftment, and differentiation into resident cells were thought, initially, to be necessary to obtain the beneficial effects of these cells, and clinical experience and several experiments have shown that one of the primary functions of MSCs, most likely their critical function, is to secrete several bioactive molecules related to the microenvironment "niche" in which these cells are located. Consequently, the secretome reproduces most of the effects of MSCs transiently; in this sense, MSCs secrete a wide variety of pro-inflammatory and anti-inflammatory cytokines, chemokines, growth factors, and prostaglandins under resting and inflammatory conditions (Hmadcha et al. 2009).

These molecules are associated with immunomodulation (indoleamine-2,3-dioxygenase (IDO), prostaglandin-E2 (PGE-2), transforming growth factor beta (TGF-β), human leukocyte antigen-G5 (HLA-G5), and hepatocyte growth factor (HGF)), anti-apoptosis (vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), TGF-β, stanniocalcin-1 (STC1), and insulin-like growth factor 1 (IGF-1)), angiogenesis (VEGF, monocyte chemoattractant protein 1 (MCP-1), and IGF-1), local stem and progenitor cell growth and differentiation support (CSF complex, angiopoietin-1, and stromal cell-derived factor 1 (SDF-1)), anti-fibrosis (HGF and basic fibroblast growth factor (bFGF)), and chemoattraction (chemokine (C-C motif) ligands 2 and 4 (CCL2, CCL4) and C-X-C motif chemokine 12 (CXCL12 also called SDF1)) (Meirelles Lda et al. 2009).

MSCs display a low expression of major histocompatibility complex class I human leukocyte antigen (MHC-HLA class I), while they are constitutively negative for HLA-class II; likewise, they do not express costimulatory molecules such as CD80, CD86, CD40, and CD40L. However, MSCs share the expression of surface markers, such as vascular cell adhesion protein 1 (VCAM-1), intercellular adhesion molecule 2 (ICAM-2), and lymphocyte function-associated antigen 3 (LFA-3 or CD58) with the thymic epithelium, which is crucial for the interaction with T cells

(Hmadcha et al. 2009; Le Blanc 2003). Whereas MSCs remain in a quiescent state showing anti-apoptotic properties and contributing to homeostasis, in an inflammatory environment (presence of IFN-γ, TNF-α, IL-1α, and IL-1β), they begin to exercise their immunomodulation abilities, inhibiting the proliferation of effector cells and their cytokine production. In the same way, MSCs can block various immune cell functions (Hmadcha et al. 2009; Cagliani et al. 2017).

There is a complex "cross-talk" interaction between MSCs and endothelial cells (ECs). MSCs increase the proliferation and migration of the ECs, promoting early events of angiogenesis and decreasing the permeability of the monolayer of the ECs. In direct co-cultures of MSCs and ECs, MSCs increased the persistence of pre-existing blood vessels in a dose-dependent manner (Duffy et al. 2009). Moreover, beneficial therapeutic effects of the use of conditioned media of MSCs have been reported; even it is therapeutically better than the cells themselves (Burlacu et al. 2013; Shrestha et al. 2013) and to stimulate the proliferation of local ECs (Potapova et al. 2007). Likewise, in addition to direct "cell-cell" contact, speculation has been made with a possible transfer of mitochondria or vesicular components (secretome) that contain mRNA, microRNA, and proteins (Tan et al. 2021). Not only have this, the exosomes, secretory extracellular vesicles (EVs) from MSCs, also been identified to produce the same immunomodulatory activity as MSCs (Haider and Aramini 2020). Targeting the MSCs' secretome as an acellular therapeutic agent could provide several advantages over the use of cell-based therapies for various diseases paving the way for cell-free therapy (Haider and Aslam 2018; Bari et al. 2019).

Altogether, these features constitute an area of research in expansion in the last decade and make MSCs an eligible therapeutic candidate to be evaluated within clinical trials for a plethora of diseases such as diabetes and diabetes complication and cardiovascular and neurological diseases; in immune-mediated disorders, such as graft-versus-host diseases (GVHD), multiple sclerosis (MS), Crohn's disease (CD), and osteoarthritis (OA); and even in immune-dysregulating infectious diseases such as the novel coronavirus disease 2019 (COVID-19) (see ▶ Chap. 6, "Mesenchymal Stromal Cells for COVID-19 Critical Care Patients," of this book for review on COVID-19).

When writing this chapter (May 2021), 1.276×10^3 publicly and privately funded clinical studies worldwide in which MSCs have been used have been reported and registered in the US National Library of Medicine database (NIH-ClinicalTrials.gov). Although the therapeutic efficacy of MSCs has been demonstrated in different disease animal models and numerous human phase 1/2 clinical trials and generally communicated, only very few (84 studies) phase 3/4 clinical trials using MSCs are registered (Table 1) and have demonstrated the expected potential therapeutic benefit. Almost all registered clinical trials are early phase 1/2 with safety as the primary objective. For efficacy and effectiveness issues, other advanced phases are mandatory. In all cases, one cannot consider these issues (efficacy nor effectiveness) unless phase 3 clinical trials are developed (García-Bernal et al. 2021) (Fig. 1).

Even though MSCs and their EVs have been shown to have high potential benefits in regenerative medicine and cell-free-based therapy, their clinical application remains controversial; thus, considerations and determination of possible side

Table 1 Public registry list of phase 3/4 clinical trials using MSCs as cell-based therapy (NIH-*ClinicalTrial.gov*)

NCT number	Title	Status	Phases	URL
NCT03106662	Mesenchymal Stem Cell Infusion in Haploidentical Hematopoietic Stem Cell Transplantation in Patients with Hematological Malignancies	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT03106662
NCT04351932	Bone Marrow Versus Adipose Autologous Mesenchymal Stem Cells for the Treatment of Knee Osteoarthritis	Not yet recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04351932
NCT02755922	Bone Regeneration with Mesenchymal Stem Cells	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT02755922
NCT04224207	Management of Retinitis Pigmentosa by Mesenchymal Stem Cells by Wharton's Jelly Derived Mesenchymal Stem Cells	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT04224207
NCT01854125	Autologous Mesenchymal Stem Cell Transplantation in Cirrhosis Patients with Refractory Ascites	Unknown status	Phase 3	https://ClinicalTrials.gov/show/NCT01854125
NCT02218437	Treatment Protocol of Child SAA with the Injection of Mesenchymal Stem Cells (Umbilical Cord Derived)	Unknown status	Phase 4	https://ClinicalTrials.gov/show/NCT02218437
NCT01716481	The STem Cell Application Researches and Trials In NeuroloGy-2 (STARTING-2) Study	Unknown status	Phase 3	https://ClinicalTrials.gov/show/NCT01716481
NCT00366145	Efficacy and Safety of Adult Human Mesenchymal Stem Cells to Treat Steroid Refractory Acute Graft Versus Host Disease	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT00366145
NCT03766217	Bone Tissue Engineering with Dental Pulp Stem Cells for Alveolar Cleft Repair	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT03766217
NCT04689152	Clinical Trial to Evaluate the Efficacy and Safety of Cellgram-LC Administration in Patients with Alcoholic Cirrhosis	Not yet recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04689152
NCT01676441	Safety and Efficacy of Autologous Mesenchymal Stem Cells in Chronic Spinal Cord Injury	Terminated	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01676441

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT01157403	Autologous Transplantation of Mesenchymal Stem Cells for Treatment of Patients with Onset of Type 1 Diabetes	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01157403
NCT03325504	A Comparative Study of 2 Doses of BM Autologous H-MSC +Biomaterial vs Iliac Crest AutoGraft for Bone Healing in Non-Union	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT03325504
NCT01652209	To Evaluate the Efficacy and Safety of Hearticelgram®-AMI in Patients with Acute Myocardial Infarction	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT01652209
NCT01873625	Transplantation of Bone Marrow Derived Mesenchymal Stem Cells in Affected Knee Osteoarthritis by Rheumatoid Arthritis	Completed	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01873625
NCT01392105	Safety and Efficacy of Intracoronary Adult Human Mesenchymal Stem Cells After Acute Myocardial Infarction	Completed	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01392105
NCT00543374	Extended Evaluation of PROCHYMAL® Adult Human Stem Cells for Treatment-Resistant Moderate-to-Severe Crohn's Disease	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT00543374
NCT01394432	ESTIMATION Study for Endocardial Mesenchymal Stem Cells Implantation in Patients After Acute Myocardial Infarction	Unknown status	Phase 3	https://ClinicalTrials.gov/show/NCT01394432
NCT02442817	Linagliptin and Mesenchymal Stem Cells: A Pilot Study	Completed	Phase 4	https://ClinicalTrials.gov/show/NCT02442817
NCT00482092	Evaluation of PROCHYMAL® Adult Human Stem Cells for Treatment-Resistant Moderate-to-Severe Crohn's Disease	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT00482092
NCT01526850	Efficacy and Safety Study of Allogenic Mesenchymal Stem Cells for Patients with Chronic Graft Versus Host Disease	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01526850
NCT04421274	Bone Marrow Mesenchymal Stem Cells Transfer in Patients with ST-Segment Elevation Myocardial Infarction	Completed	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04421274

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT03818737	Multicenter Trial of Stem Cell Therapy for Osteoarthritis (MILES)	Active, not recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT03818737
NCT02223897	Mesenchymal Stem Cells Transplantation for Ischemic-Type Biliary Lesions	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02223897
NCT00891501	The Use of Autologous Bone Marrow Mesenchymal Stem Cells in the Treatment of Articular Cartilage Defects	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT00891501
NCT01873547	Different Efficacy Between Rehabilitation Therapy and Stem Cells Transplantation in Patients with SCI in China	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01873547
NCT04146519	Parkinson's Disease Therapy Using Cell Technology	Recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04146519
NCT04243681	Combination of Autologous MSC and HSC Infusion in Patients with Decompensated Cirrhosis	Completed	Phase 4	https://ClinicalTrials.gov/show/NCT04243681
NCT04297813	Efficacy in Alveolar Bone Regeneration with Autologous MSCs and Biomaterial in Comparison to Autologous Bone Grafting	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04297813
NCT02334878	Stem Cell Therapy for Treatment of Female Stress Urinary Incontinence	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT02334878
NCT01759212	Left Ventricular Assist Device Combined with Allogeneic Mesenchymal Stem Cells Implantation in Patients with End-Stage Heart Failure	Active, not recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01759212
NCT04877067	Therapy of Toxic Optic Neuropathy via Combination of Stem Cells with Electromagnetic Stimulation	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT04877067
NCT04366063	Mesenchymal Stem Cell Therapy for SARS-CoV-2-Related Acute Respiratory Distress Syndrome	Recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04366063
NCT03990805	A Phase 3 Study to Evaluate the Efficacy and Safety of JointStem in Treatment of Osteoarthritis	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT03990805

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT02672267	A Study of Allogeneic Low Oxygen Mesenchymal Bone Marrow Cells in Subjects with Myocardial Infarction	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT02672267
NCT00851162	Using Mesenchymal Stem Cells to Fill Bone Void Defects in Patients with Benign Bone Lesions	Withdrawn	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT00851162
NCT01803347	Clinical Trial to Evaluate the Efficacy and Safety of Stem Cells	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01803347
NCT04612465	Clinical Study to Evaluate Efficacy and Safety of ASC and Fibringlue or Fibringlue in Patients with Crohn's Fistula	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04612465
NCT02241018	MSCs Combined with CD25 Monoclonal Antibody and Calcineurin Inhibitors for Treatment of Steroid-Resistant aGVHD	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02241018
NCT04427930	Follow-Up Study for Participants of JointStem Phase 3 Clinical Trial	Enrolling by invitation	Phase 3	https://ClinicalTrials.gov/show/NCT04427930
NCT03633565	Comparative Study of Strategies for Management of Duchenne Myopathy (DM)	Not yet recruiting	Phase 4	https://ClinicalTrials.gov/show/NCT03633565
NCT03389919	Intraosseous Administration of Mesenchymal Stromal Cells for Patients with Graft Failure After Allo-HSCT	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT03389919
NCT04368806	A Phase 2b/3a Study to Evaluate the Efficacy and Safety of JointStem in Patients Diagnosed as Knee Osteoarthritis	Not yet recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04368806
NCT03370874	Clinical Study to Evaluate Efficacy and Safety of ALLO-ASC-DFU in Patients with Diabetic Foot Ulcers	Active, not recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT03370874
NCT03631589	MSC for Severe aGVHD	Recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT03631589
NCT03280056	Safety and Efficacy of Repeated Administrations of NurOwn® in ALS Patients	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT03280056

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT04738981	Efficacy and Safety of UC-MSCs for the Treatment of Steroid-Resistant aGVHD Following Allo-HSCT	Not yet recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04738981
NCT02809781	A Pilot Study of MSCs Iufusion and Etanercept to Treat Ankylosing Spondylitis	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02809781
NCT01041001	Study to Compare Efficacy and Safety of Cartistem and Microfracture in Patients with Knee Articular Cartilage Injury	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01041001
NCT02240992	MSCs With or Without Peripheral Blood Stem Cell for Treatment of Poor Graft Function and Delayed Platelet Engraftment	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02240992
NCT01541579	Adipose Derived Mesenchymal Stem Cells for Induction of Remission in Perianal Fistulizing Crohn's Disease	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01541579
NCT03905824	The Effectiveness of Adding Allogenic Stem Cells After Traditional Treatment of Osteochondral Lesions of the Talus	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT03905824
NCT01233960	Evaluation of PROCHYMAL® for Treatment-Refractory Moderate-to-Severe Crohn's Disease	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01233960
NCT02291770	Treatment of Chronic Graft-Versus-Host Disease with Mesenchymal Stromal Cells	Unknown status	Phase 3	https://ClinicalTrials.gov/show/NCT02291770
NCT01929434	Efficacy of Stem Cell Transplantation Compared to Rehabilitation Treatment of Patients with Cerebral Paralysis	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01929434
NCT01759823	Bone Marrow Derived Stem Cell Transplantation in T2DM	Completed	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT01759823
NCT01626677	Follow-Up Study of CARTISTEM® Versus Microfracture for the Treatment of Knee Articular Cartilage Injury or Defect	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01626677

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT03404063	Cardiovascular Clinical Project to Evaluate the Regenerative Capacity of CardioCell in Patients with Acute Myocardial Infarction (AMI)	Completed	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT03404063
NCT04219241	Clinical Extension Study for Safety and Efficacy Evaluation of Cellavita-HD Administration in Huntington's Patients	Not yet recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04219241
NCT04018729	Cell Therapy Associated with Endobronchial Valve	Not yet recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04018729
NCT04230902	Effects of α MAT Versus Steroid Injection in Knee Osteoarthritis (STα MAT-Knee Study)	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04230902
NCT03418233	Randomized Clinical Trial to Evaluate the Regenerative Capacity of CardioCell in Patients with Chronic Ischaemic Heart Failure (CIHF)	Completed	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT03418233
NCT03423732	Cardiovascular Clinical Project to Evaluate the Regenerative Capacity of CardioCell in Patients with No-option Critical Limb Ischemia (N-O CLI)	Active, not recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT03423732
NCT03112122	Study for the Treatment of the Bone Marrow Edema:Core Decompression VS Bone Marrow Concentrate (BMC) VS Bone Substitute	Terminated	Phase 4	https://ClinicalTrials.gov/show/NCT03112122
NCT02138331	Effect of Microvesicles and Exosomes Therapy on β-Cell Mass in Type I Diabetes Mellitus (T1DM)	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02138331
NCT03042572	Allogeneic Mesenchymal Stromal Cells for Angiogenesis and Neovascularization in No-option Ischemic Limbs	Not yet recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT03042572
NCT04247945	Co-transplantation of MSC in the Setting of Allo-HSCT	Recruiting	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04247945
NCT04745299	Evaluation the Efficacy and Safety of Mutiple Lenzumestrocel (Neuronata-R® Inj.) Treatment in patients with ALS	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04745299

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT04126603	Impact of Semaglutide on CD34 + EPC and Fat Derived MSC	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04126603
NCT04371393	MSCs in COVID-19 ARDS	Active, not recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04371393
NCT04310215	Efficacy and Safety of Allogenic Stem Cell Product (CARTISTEM®) for Osteochondral Lesion of Talus	Enrolling by invitation	Phase 3	https://ClinicalTrials.gov/show/NCT04310215
NCT04138017	ViviGen Cellular Bone Matrix for Hindfoot or Ankle Arthrodesis	Enrolling by invitation	Phase 4	https://ClinicalTrials.gov/show/NCT04138017
NCT03747822	Evaluation of Soft Tissue Profile Changes Following Autogenous Fat or Onlay Polyetheretherketone (PEEK) Augmentation Versus Sliding Genioplasty for Correction of Deficient Chin	Unknown status	Phase 3	https://ClinicalTrials.gov/show/NCT03747822
NCT04569409	Clinical Study to Evaluate Efficacy and Safety of ALLO-ASC-DFU in Patients with Diabetic Wagner Grade 2 Foot Ulcers	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04569409
NCT01926327	The Effect of Platelet-Rich Plasma in Patients with Osteoarthritis of the Knee	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT01926327
NCT04864509	The Effects of Melatonin Treatment on Bone, Marrow, Sleep and Blood Pressure	Not yet recruiting	Phase 4	https://ClinicalTrials.gov/show/NCT04864509
NCT02448849	Autologous BM-MSC Transplantation in Combination with Platelet Lysate (PL) for Nonunion Treatment	Unknown status	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02448849
NCT04541680	Nintedanib for the Treatment of SARS-Cov-2 Induced Pulmonary Fibrosis	Recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04541680
NCT01529008	Study on Autologous Osteoblastic Cells Implantation to Early Stage Osteonecrosis of the Femoral Head	Terminated	Phase 3	https://ClinicalTrials.gov/show/NCT01529008

(continued)

Table 1 (continued)

NCT number	Title	Status	Phases	URL
NCT00562497	Efficacy and Safety of Prochymal™ Infusion in Combination with Corticosteroids for the Treatment of Newly Diagnosed Acute Graft Versus Host Disease (GVHD)	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT00562497
NCT02849613	Regenerative Stem Cell Therapy for Stroke in Europe	Withdrawn	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT02849613
NCT04629833	Treatment of Steroid-Refractory Acute Graft-Versus-Host Disease with Mesenchymal Stromal Cells Versus Best Available Therapy	Not yet recruiting	Phase 3	https://ClinicalTrials.gov/show/NCT04629833
NCT02336230	A Prospective Study of Remestemcel-L, Ex-Vivo Cultured Adult Human Mesenchymal Stromal Cells, for the Treatment of Pediatric Patients Who Have Failed to Respond to Steroid Treatment for Acute GVHD	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT02336230
NCT02032004	Efficacy and Safety of Allogeneic Mesenchymal Precursor Cells (Rexlemestrocel-L) for the Treatment of Heart Failure	Completed	Phase 3	https://ClinicalTrials.gov/show/NCT02032004

effects need to be addressed to optimize the clinical application of this double-edged sword cellular medicament. This chapter updates and discusses the state of the art in MSCs' cell-based therapies and provides relevant information regarding factors to consider for the clinical application of MSCs.

Biological Characteristics

Phenotypic Profile

Since Friedenstein and colleagues first isolated a colony-forming unit fibroblast (CFU-F) from BM, BM has been widely used as a source of MSCs for many investigations and clinical trials. In addition to BM (BM-derived MSCs), MSCs have been isolated from different tissues, such as adipose tissue (Ad-MSCs), umbilical cord (UCB-MSCs), dental pulp, synovial liquid, and amniotic fluid. All these tissues vary in their cellular components, signals, and factors secreted, resulting in different immediate microenvironment conditions, thus developing

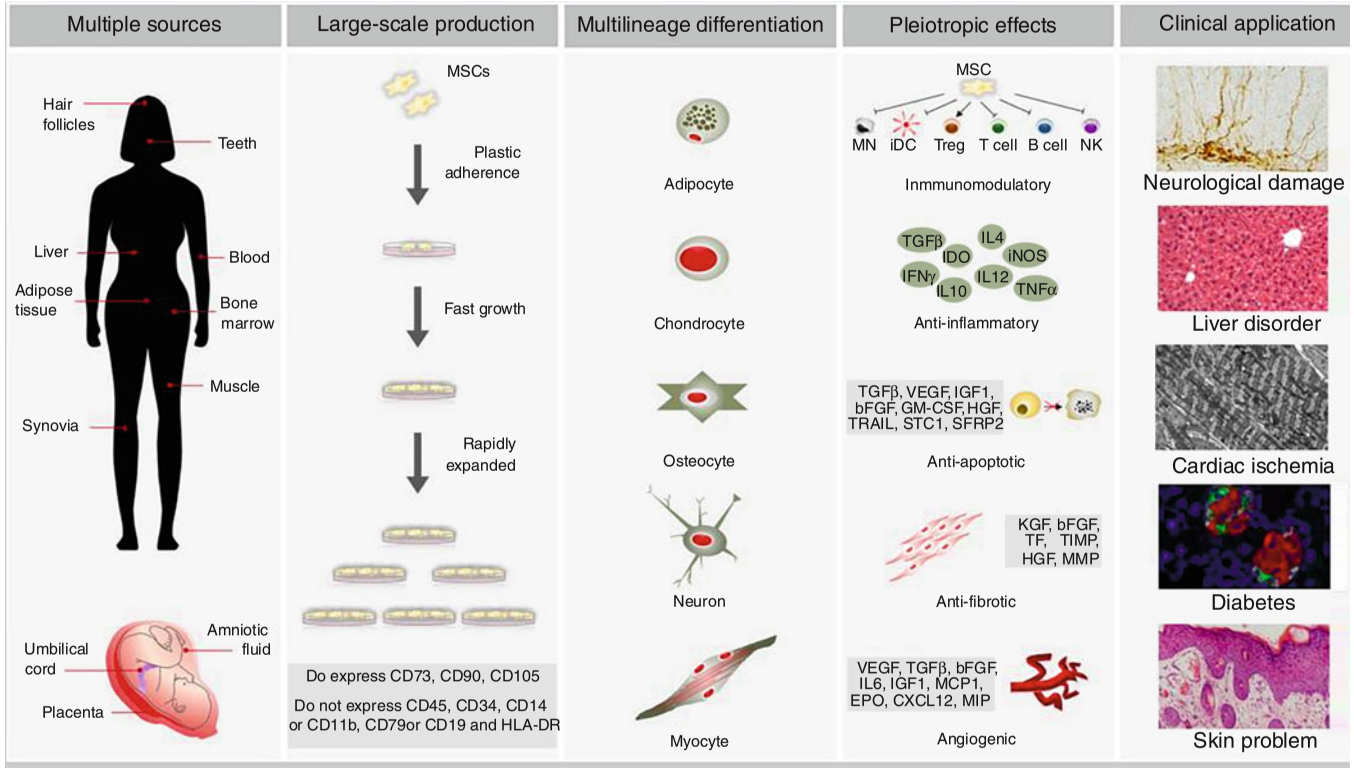

Fig. 1 The MSCs have multiple potential advantages for their clinical application. Among other advantages, MSCs can be isolated from various sources, are produced on a large scale, differentiate into various cell types, and have pleiotropic effects. Such advantages make MSCs suitable for clinical application in different disease conditions, such as neurological damage, liver disorders, cardiac ischemia, diabetes, or skin problems. (Re-published from Hmadcha 2020,

several physiological niches (Hmadcha et al. 2009; Escacena et al. 2015). Although isolated and long-term cultured MSCs of most tissues show similar immunophenotypic characteristics, some differences have been found among MSCs of different tissue origins according to data obtained by in vitro experiments. In 2006, the International Society for Cellular Therapy (ISCT) published the minimal criteria to define MSCs by nomenclature and by biological characteristics to allow studies from different groups to be compared and contrasted. These criteria include the co-expression of markers such as CD73, CD90, and CD105, a lack of expression of hematopoietic markers (CD45, CD34, and CD14) and HLA-DR, multipotent differentiation potential, and adherence to plastic (Horwitz et al. 2005). However, several researchers have noted that Ad-MSCs express CD34 and CD54 in early passages and have lower expression of CD106 and that umbilical cord blood-derived MSCs (UCB-MSCs) express CD90 and CD105. Other markers have been used in different studies, and other differences have emerged, such as VEGFR-2 (Flk-1) expression, which was significantly higher in periosteum-derived cells than in adipose tissue- and muscle-derived cells, or the rate of NGFR positivity, which was much higher in muscle-derived cells than in other mesenchymal tissue-derived cells (Escacena et al. 2015).

Although some immunophenotypic differences have been documented, many researchers consider that these differences could be due to distinct extraction methods and different culture methodologies, resulting in variations of MSC surface markers. Therefore, this chapter aimed to investigate markers and characteristics that are more specific to select better sources of MSCs for clinical applications. Likewise, expanding the cells in vitro is necessary to obtain the desired numbers for therapeutic approaches. Changes in the proteomic phenotype of MSCs have been observed during high passages, although no proper approaches to examine the state of cells continuously during long-term in vitro culture have been established (Capra et al. 2012). Some researchers ascribe these variations to the adaptation of cells to the environment; thus, determining the biomolecular markers that are involved in these variations is essential for obtaining a better phenotypic characterization of these cells and thus for achieving more effective cell therapy in the future (Escacena et al. 2015).

Fig. 1 (continued) article published under CC-BY terms). Abbreviations: bFGF basic fibroblast growth factor, CXCL12 C-X-C motif chemokine 12, EPO erythropoietin, GM-CSF granulocyte-macrophage colony-stimulating factor, HGF hepatocyte growth factor, HLA-DR major histocompatibility complex class II DR, iDC immature dendritic cell, IDO indoleamine-2,3-dioxygenase, IGF1 insulin-like growth factor 1, IL-10 interleukin-10, IL-12 interleukin-12, IL-4 interleukin-4, IL-6 interleukin-6, INF-γ interferon-γ, iNOS inducible nitric oxide synthase, KGF keratinocyte growth factor, MCP1 monocyte chemoattractant protein 1, MIP macrophage inflammatory protein, MMP matrix metalloproteinases, MN monocyte, NK natural killer cell, SFRP2 secreted frizzled-related protein 2, STC1 stanniocalcin 1, TF tissue factor, TGF-β transforming growth factor beta, TIMP tissue inhibitor of metalloproteinases, TNF-α tumor necrosis factor α, TRAIL TNF-related apoptosis-inducing ligand, Treg regulatory T cell, VEGF vascular endothelial growth factor

MSCs' Proliferation

The proliferative activity of MSCs is another feature that may be affected by the different origins of MSCs. The rate and persistence of MSC proliferation appear to vary between source tissues. MSCs are considered adult stem cells, and unlike embryonic stem cells (ESCs), these cells have a limited proliferative capacity. Physiological niches maintain adult stem cells in an undifferentiated state; however, when MSCs are cultured in vitro, they age, which affects their therapeutic properties, such as alterations in phenotype, differentiation potential, global gene expression patterns, miRNA profiles, and even chromosomal abnormalities, particularly after long-term culture or when cells of multiple doublings are used (Escacena et al. 2015). Large numbers of MSCs are needed for therapeutic applications, and in vitro expansion is required to produce the desired MSC numbers. In vivo, MSCs represent 0.0001% of nucleated BM cells, and their number decreases with the donor's age. The quantity of MSCs (CFU-Fs) among nucleated BM cells decreases with age from one MSC in 10^4 BM cells in newborn to one MSC in 10^5 cells in teenagers and one MSC in 10^6 cells in older individuals (Caplan 2009).

Furthermore, MSCs from older human donors differ significantly from younger donors in morphology, replicative lifespan, doubling time, healing capacity, and differentiation potential. Sufficient evidence has indicated that MSCs from older donors have limited therapeutic efficacy. Some studies have suggested that the difference between preclinical and clinical findings is due to the donor age (Stenderup et al. 2003; Escacena et al. 2015). Therefore, considering that several age-related diseases exist and that elderly patients are potential users of cell therapy, understanding the molecular and biological effects of aging on MSCs is essential for developing safe and effective MSC-based autologous cell therapy. Meanwhile, the use of allogeneic MSCs may be a treatment option for these specific patients. As commented below, MSCs elude allogeneic rejection, and their infusion is feasible and well-tolerated, with no adverse effects (McAuley et al. 2014; Liang et al. 2010).

Differentiation Capacity

MSCs can differentiate in vitro into several mesenchymal lineages, including adipose tissue, bone, cartilage, and muscle (Pittenger et al. 1999; Prockop 1997; Bruder et al. 1997). Furthermore, MSCs can differentiate into ECs, neurons, and glial cells because MSCs express genes related to specific lineages rather than those of the mesenchymal lineage (Woodbury et al. 2002). Although multilineage differentiation is another minimal criterion advised by the ISCT and undoubtedly represents a fundamental property of MSCs, this ability depends primarily on the source tissue from which these cells are derived. As such, Sakaguchi and colleagues (Sakaguchi et al. 2005) compared human MSCs isolated from BM, synovium, periosteum, skeletal muscle, and adipose tissue. The cells were expanded by similar processes; synovium-derived cells had the most remarkable ability for chondrogenesis; adipose- and synovium-derived cells, for adipogenesis; and BM-, synovium-, and

periosteum-derived cells, for osteogenesis. In another comparative analysis, UCB-MSCs showed no adipogenic differentiation capacity compared to BM- and Ad-MSCs (Kern et al. 2006).

As discussed by Horwitz (Horwitz et al. 2002), who used differentiated MSCs in a study to test the regeneration of damaged tissues, BM-derived MSCs can engraft after transplantation, differentiate to functional osteoblasts, and contribute to the formation of new dense bone in children with osteogenesis imperfecta. Most likely, the microenvironment in which MSCs are transplanted directly influences in their distinct differentiation pathways. New insights into the biological characteristics of MSCs are needed to achieve future therapies.

Cellular Transformation

In general, successive passages or long-term cultures induce genetic instability and cell transformation. Several authors have described that MSCs cultivated in vitro can be expanded multiple times without an apparent loss of differentiation potential or chromosomal alterations and even that long-term MSC cultures can develop chromosomal abnormalities but without an obvious potential for transformation (Koç et al. 2000; Le Blanc et al. 2004; Ringdén et al. 2006; Fang et al. 2006; Ning et al. 2008). Although no tumor formation in humans has been reported after the administration of MSCs, several factors must be considered that can contribute significantly to the induction of cytogenetic abnormalities, such as aspects related to the manufacturing process of the cellular medicine (e.g., culture conditions and duration of cell expansion) and heterogeneity of the MSC population (e.g., cells in different stages of duplication). The tumorigenic potential of a cell therapy medicament may depend on intrinsic and extrinsic factors, such as the administration site in the patient (due to the receptor's microenvironment) and/or the manipulation of the culture ex vivo.

Mechanism of Action

Cell Migration Toward Damaged Tissues

The success of an advanced therapy medicinal product initially depends on its ability to reach target tissues. MSCs possess inherent tropism toward damaged sites controlled by many factors and mechanisms, including chemoattractant signals. For instance, the C-X-C motif chemokine ligand 12 (CXCL12) is a frequent triggering factor at the injury site. It has been demonstrated that a subpopulation of MSCs expresses the C-X-C chemokine receptor type 4 (CXCR4) that binds to its ligand, the CXCL12, to mediate cell migration (Wynn et al. 2004; Ma et al. 2015). Aside from CXCR4, MSCs express other chemokine receptors, such as CCR1, CCR2, CCR4, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, and CX3CR1 (Sordi et al. 2005; Von Lüttichau et al. 2005; Honczarenko

et al. 2006; Ringe et al. 2007). These receptors are essential to respond to triggering factors at the site of injury. In addition, MSCs also express cell adhesion molecules, including CD49d, CD44, CD54, CD102, and CD106 (De Ugarte et al. 2003). These chemokines and cell adhesion molecules orchestrate the mobilization of MSCs' injury sites in a similar manner to white blood cells (Kolaczkowska and Kubes 2013). MSC mobilization is a multistep process that encompasses the attachment of free circulating MSCs in the bloodstream to transmigrate between ECs with the ultimate goal of migrating and engrafting to the target tissue.

Tissue Repair

Once recruited in the injured site, MSCs contribute to tissue repair and regeneration by activating several mechanisms. A growing body of research has demonstrated that MSCs display pleiotropic effects, which give them enormous therapeutic potential. MSCs secrete various mediators of tissue repair in response to injury signals, including anti-apoptotic, anti-inflammatory, immunomodulatory, anti-fibrotic, and angiogenic agents (Caplan and Dennis 2006; Meirelles Lda et al. 2009; Maltman et al. 2011; Escacena et al. 2015). Among pleiotropic effects, anti-inflammatory and immunomodulatory properties are mainly responsible for the therapeutic benefits of MSCs. As sensors of inflammation, MSCs release soluble factors, such as TGF-β, IDO, TNF-α, IL-10, and INF-γ, which interfere with the immune system and modify the inflammatory landscape (Prockop and Oh 2012). Pivotal studies showed that MSCs inhibit the proliferation of T and B cells (Di Nicola et al. 2002; Corcione et al. 2006; Song et al. 2019), suppress the activation of natural killer cells (Sotiropoulou et al. 2006), and prevent the generation and maturation of monocyte-derived dendritic cells (English et al. 2008; Spaggiari et al. 2009). Furthermore, MSCs can promote the generation of regulatory T cells (Maccario et al. 2005), which exert immunosuppressive effects. Although soluble factors play a key role in the immunosuppressive activity of MSCs, cell-to-cell contact also influences immune responses (Ren et al. 2010; Li et al. 2019). For instance, direct contact between MSCs and pro-inflammatory macrophages has been shown to induce immune tolerance by inducing tumor necrosis factor-stimulated gene-6 (TSG-6) production (Li et al. 2019). MSC mediated modulations of the immune response set in motion essential inflammatory processes that significantly promote tissue repair and regeneration by driving healing, scarring, and fibrosis (Julier et al. 2017).

Immunomodulatory Potential

The immunomodulatory properties of MSCs and their immune-privileged condition make these cells good candidates for use in several clinical trials related to chronic, inflammatory, and autoimmune diseases. MSCs interact with cells of the innate or

adaptive immune system (T cells, B cells, NK cells, monocyte-derived dendritic cells, and neutrophils) (Di Nicola et al. 2002; Raffaghello et al. 2008). For a cell to be recognized by the immune system, the expression of major histocompatibility complex (MHC) and co-stimulatory molecules is necessary. MHC class I and class II human leukocyte antigens (HLAs) are master triggers of robust immunological rejection of grafts because they present antigens to cytolytic T lymphocytes (CTL). Human mesenchymal stem cells (hMSCs) are characterized by low expression of MHC class I HLAs but are constitutively negative for class II HLCs; these cells do not express co-stimulatory molecules such as B7-1, B7-2, CD80, CD86, CD40, and CD40L (Hmadcha et al. 2009; Le Blanc 2003). However, similar to the thymic epithelium, MSCs express the surface markers VCAM-1, ICAM-2, and LFA-3 (Le Blanc 2003; Conget and Minguell 1999), which are crucial for T-cell interactions.

Although a T-cell response should be expected, hMSCs can modulate the activation and proliferation of both $CD4^+$ and $CD8^+$ cells in vitro by arresting T cells in G0/G1 phase (Glennie et al. 2005; Benvenuto et al. 2007). Different studies have suggested that cell-cell interactions and certain soluble factors are the mechanisms used by MSCs to mediate the immune response. Factors, such as IDO, TGF-β1, IFN-γ, IL-1β, TNF-α, IL-6, IL-10, PGE-2, HGF, HLA-G5, and others, are secreted by MSCs or released after interactions with target cells. As mentioned above, MSCs remain in a resting state, display anti-apoptotic properties, and maintain different cells such as hematopoietic stem cells (HSCs), thus contributing to tissue homeostasis. However, in an inflammatory environment such as that created by cytokines such as IFN-γ, TNF-α, IL-1α, and IL-1β, MSCs begin to exert their immunosuppressive effects and polarize, inhibiting the proliferation of effector cells and their production of cytokines. In this regard, IFN-γ is postulated as a "licensing" agent for MSC anti-proliferative action. MSCs may also acquire behavior as antigen-presenting cells (APCs) under specific concentrations of IFN-γ (Stagg et al. 2006; Uccelli et al. 2008). However, no consensus regarding what concentration of IFN-γ is more necessary for MSCs to show inhibitory or APC functions exists.

Likewise, TNF-α is another pro-inflammatory cytokine involved in the MSC immune response, and TNF-α enhances the effect of IFN-γ. IFN-γ, with or without the help of TNF-α, stimulates the production of IDO by MSCs, inhibiting the proliferation of activated T or NK cells and thus enhancing the homing potential and reparative properties of these cells; however, some potential risks are associated with the role of IFN-γ (Krampera et al. 2006; Sivanathan et al. 2014). Some authors have maintained that the immunomodulatory properties of MSCs are comparable, while others have argued that MSCs of different tissue origins or species cannot have equivalent and comparable immunomodulatory properties (Najar et al. 2010; Yoo et al. 2009; Ricciardi et al. 2012; Krampera 2011). For example, MSCs from perinatal sources (umbilical cord and amniotic membrane) show a higher immunomodulatory capacity, differential gene expression profiles, and paracrine factor secretion compared to BM-MSCs (Wegmeyer et al. 2013). Lee and colleagues found that HLA-G, a specific MHC-I antigen that is critical for maintaining the

immune-tolerant state of pregnancy and that is a contributing factor to the induction of more substantial immunosuppression, is strongly positive only in placenta-derived MSCs (PD-MSCs) (Lee et al. 2012a). This is in contrast to BM-derived MSCs and Ad-MSCs and suggests that the immunophenotype of PD-MSCs may be superior to other MSCs in terms of their immunosuppressive function (Hunt et al. 2005). Nonetheless, some authors claimed that BM-derived MSCs were more immunomodulatory than PD-MSCs (Fazekasova et al. 2011). And others concluded that the immunomodulatory capacities of BM-derived MSCs and Ad-MSCs are similar but that differences in cytokine secretion cause Ad-MSCs to have more potent immunomodulatory effects than BM-derived MSCs (Melief et al. 2013).

Bartholomew and colleagues (Bartholomew et al. 2002) showed that allogeneic MSCs prolonged skin graft survival in baboons. Mouse MSCs have been used in related experiments; these cells use inducible nitric oxide synthase (iNOS) for immunosuppression instead of IDO. These findings indicate that MSCs differ between species (Ren et al. 2009). Since then, several preclinical models have been used to analyze the biological effects of MSCs and their ability to modulate immune responses, considering that not all animal models mimic human diseases. Once more, these differences could be due to isolation procedures, to culture methodology, or, more likely, to differences in the microenvironments where cells reside. These and other findings lead us to conclude that determining whether these differences may be relevant for clinical applications and whether MSCs of a particular tissue type are more appropriate for specific therapies or diseases.

Preclinical Applications

Preclinical models are essential for clinicians, researchers, and both national and international regulatory agencies to demonstrate the safety and efficacy of MSC-based therapies (Krampera et al. 2013). Because MSCs can exert immunomodulatory properties and act on different immune cells in vitro and in vivo, these cells have begun to be used against autoimmune diseases based on multiple autoimmune experimental models. Pioneer studies in experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, reported that MSCs derived from numerous tissue origins show efficacy against neurodegenerative disorders (Zappia et al. 2005; Rafei et al. 2009; Constantin et al. 2009; Bai et al. 2009; Zhang et al. 2005). BM-MSC and UCB-MSC treatments have improved clinical and laboratory parameters in systemic lupus erythematosus (SLE) (Sun et al. 2010). Furthermore, ameliorating effects have been observed in experimental mouse models of rheumatoid arthritis (RA) (González et al. 2009). Diabetes is another autoimmune disorder in which MSCs have been employed (Jurewicz et al. 2010; Lee et al. 2006). Although promising results and progress have been observed in this field, the interspecies differences, and contradictory experimental outcomes, and the inability to recreate the complete pathophysiology of some diseases make it necessary to search for new animal models for comparable results.

MSC-Based Therapy for Autoimmune Diseases

The MSCs are being used to facilitate the engraftment of transplanted HSCs and treat graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (HSCT) based on their immunomodulatory properties and provide appropriate conditions. However, preclinical and clinical experiments with MSCs do not always show similar results for the prevention and treatment of GvHD. In a study using a mouse model of GvHD (Sudres et al. 2006), MSCs suppressed alloantigen-induced T-cell proliferation in vitro in a dose-dependent manner but yielded no clinical benefit regarding the incidence or severity of GvHD. Instead, when UCB-MSCs were administered in weekly doses in a xenogenic model of GvHD, a marked decrease in human T-cell proliferation was observed, and none of the mice developed GvHD. No therapeutic effect was obtained when UCB-MSCs were administered at the onset of GvHD (Tisato et al. 2007). In the same line of research, serial infusions of mouse AD-MSCs could efficiently control the lethal GvHD that occurred in recipients transplanted with haploidentical hematopoietic grafts (Yañez et al. 2006). Mixed results have also been achieved in human patients. One study found that the co-transplantation of culture-expanded MSCs and HSCs from HLA-identical sibling donors after myeloablative therapy accelerated hematopoietic engraftment (Lazarus et al. 2005); however, a significant reduction of GvHD symptoms was not shown, although the incidence or severity of GvHD did not increase. Koç and colleagues (Koç et al. 2000) reported a positive impact of MSCs on hematopoiesis; rapid hematopoietic recovery was observed in a clinical study with breast cancer patients who received autologous HSCT together with autologous MSCs.

Therapeutic effects have also been reported at the onset of GvHD, such as the case of a 9-year-old boy with severe treatment-resistant GvHD after allogeneic HSCT for acute lymphocytic leukemia who received haploidentical MSCs derived from his mother. He showed improvement after two administrations of MSCs (Le Blanc et al. 2004). Similar results have been obtained in steroid-refractory GvHD pilot studies with BM-MSCs and AD-MSCs (Ringdén et al. 2006; Fang et al. 2006). Several infusions appear to be required to maintain the level of active immunomodulation by MSCs. Similarly, the expression of pro-inflammatory cytokines such as IFN-γ in the environment at the time of MSC administration is required by these cells to exert their immunosuppressive effect. A lack of MSC "licensing" can result in the absence of the desired therapeutic effect.

While evidence that MSCs are effective in combination or after HSCT in specific hematological and non-hematological diseases has been shown, adverse reactions and risk factors intrinsic to this practice have been reported. In a pilot study, HLA-identical sibling-matched HSCs were transplanted with or without MSCs in hematological malignancy patients. Although MSCs were well-tolerated and this treatment effectively prevented GVHD, six patients (60%) in the MSC group and three (20%) in the non-MSC group had 3-year disease-free survival rates of 30 and 66.7%, respectively. The relapse rate in the experimental group was higher than that

in the control group, suggesting that MSCs may impair the therapeutic graft-versus-leukemia (GVL) effect (Ning et al. 2008). In vitro and in vivo studies regarding the relationship between the immunosuppressive properties of MSCs and the stimulation of cancer growth have been performed. Mouse MSCs from the BM, spleen, and thymus injected together with a genetically modified tumor cell vaccine could equally prevent the onset of an anti-tumor memory immune response, thus leading to tumor growth in normally resistant mice (Krampera et al. 2007). In another in vivo experiment with a murine melanoma tumor model, the authors observed that the subcutaneous injection of B16 melanoma cells led to tumor growth in allogeneic recipients only when MSCs were co-injected (Djouad et al. 2003). The functions of MSCs can be influenced by the existing microenvironment, making them acquire supportive properties toward cancer cells and decrease immune reactions (Galiè et al. 2008). Therefore, potential risks related to the growth support and enhancement of undetected or "resident" cancer exist, and the administration of MSCs in these patients must be thoroughly evaluated.

MSCs for Cancer Treatment

The therapeutic benefits of MSCs have prompt their use in cell-based strategies to treat different diseases, including cancer (Hmadcha et al. 2020). Similar to damaged tissues, tumors exert chemoattractant effects on MSCs that influence their recruitment to tumor sites. The CXCL12/CXCR4 axis is one of the most frequently studied signaling pathways in mobilizing MSCs to the tumor microenvironment (Gao et al. 2009; Xu et al. 2009; Lourenco et al. 2015; Wobus et al. 2015; Kalimuthu et al. 2017). However, the ability of MSCs to migrate toward cancerous tissue is also controlled by other agents, including diffusible cytokines, such as IL-8, growth factors such as TGF-β1 or platelet-derived growth factor (PDGF), and extracellular matrix molecules, such as matrix metalloproteinase 2 (MMP-2) (Nakamizo et al. 2005; Birnbaum et al. 2007; Bhoopathi et al. 2011). Once the tumor niche is reached, MSCs interact with cancer cells via direct and indirect mechanisms that affect tumor development. The paracrine action of MSCs is one of the main mechanisms involved in cancer regulation and is mediated by multiple factors, including growth factors and cytokines. These paracrine factors affect cellular processes involving the tumor cell cycle (e.g., cell proliferation), cell survival, angiogenesis, and immunosuppression/immunomodulation, allowing MSCs to regulate cancer.

The paracrine agents can be directly secreted into the extracellular space or packaged into EVs for spreading in the tumor milieus (Rani et al. 2015). The interaction of MSCs with the tumor cell cycle is the most commonly accepted process by which MSCs exert their therapeutic effects (Fathi et al. 2019). By inhibiting proliferation-related signaling pathways, such as the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), MSCs can induce cell cycle arrest and reduce cancer growth (Lu et al. 2019). In addition, MSCs can undergo differentiation into other cell types, such as cancer-associated fibroblasts (CAFs), to directly

contribute to cancer progression (Jotzu et al. 2011; Barcellos-de-Souza et al. 2016; Aoto et al. 2018).

Accumulating evidence indicates that the cross-talk between MSCs and tumor cells results in both pro-tumor and anti-tumor effects, raising safety concerns for clinical application in oncology (Barkholt et al. 2013). The discrepancies in the ability of MSCs to promote or suppress tumor development may be attributable to differences in experimental tumor models, MSC tissue source, dose or timing of the MSC treatment, cell delivery method, control group chosen, and other experimental conditions (Bortolotti et al. 2015; Bajetto et al. 2017). In this regard, a study demonstrated that direct (cell-to-cell contact) or indirect (released soluble factors) interaction between umbilical cord MSCs and glioblastoma stem cells produces divergent effects on cell growth, invasion, and migration (Bajetto et al. 2017). Moreover, the application of MSCs for cancer patients is a more complex situation in which other factors have to be taken into consideration. For instance, the pathological conditions of each patient may induce cellular and molecular changes in MSCs that interfere with their therapeutic effects (Capilla-González et al. 2018; Pérez et al. 2018; Rivera et al. 2019). Therefore, it is important to be cautious while drawing conclusions from a single study regarding the therapeutic effects of MSCs in cancer.

The Anti-tumor Activity of MSCs

Although compelling evidence shows a pro-tumorigenic role of MSCs, these cells also have potent tumor-suppressive effects that have been exploited as cancer therapeutics. Previous studies have demonstrated that MSCs release cytotoxic agents, such as TNF-related apoptosis-inducing ligand (TRAIL) that selectively induces apoptosis in different types of cancer (Wiley et al. 1995; Hao et al. 2001; Takeda et al. 2001; Akimoto et al. 2013). Recently, a report indicated that BM-derived MSCs promote apoptosis and suppress the growth of glioma U251 cells through downregulation of the PI3K/AKT signaling pathway (Lu et al. 2019). Likewise, intravenously transplanted MSCs were found to suppress tumor growth by blocking AKT activation in a Kaposi sarcoma mouse model (Khakoo et al. 2006). In mammary carcinomas, umbilical cord MSCs attenuated cell growth and triggered apoptosis through inhibiting ERK1/2 and AKT activation (Ganta et al. 2009). The Wnt signaling pathway has also been involved in the ability of MSCs to inhibit tumor cell proliferation (Qiao et al. 2008a, b). A mechanistic study of the inhibitory effect of MSCs on breast cancer cells demonstrated that the protein dickkopf-1 (Dkk-1) released from MSCs blocks tumor growth via depression of Wnt signaling (Qiao et al. 2008a). In contrast to investigations describing the pro-angiogenic effect of MSCs (Zhang et al. 2013; Li et al. 2016), the anti-tumor activity of MSCs via the inhibition of tumor angiogenesis has also been documented. A study reported that BM-derived MSCs restrict vascular growth in 1Gli36 glioma xenograft through the downregulation of the PDGF/PDGFR axis (Ho et al. 2013). In particular, the expression of PDGF-BB protein was significantly reduced in tumor lysates when

treated with MSCs, which correlated with reduced levels of activated PDGFR-b and the active isoform of its downstream target AKT (Ho et al. 2013).

In a melanoma mouse model, transplanted MSCs inhibited angiogenesis in a concentration-dependent manner, leading to reduced tumor growth (Otsu et al. 2009). Confirmatory in vitro studies suggested that the anti-angiogenic effect was due to MSC-induced capillary degeneration (Otsu et al. 2009). Furthermore, MSCs have elicited anti-tumor immune responses through released inflammatory mediators, such as the multifunctional cytokine TGF-β. Like several signaling molecules, TGF-β plays a dual role in cancer development (Bierie and Moses 2006). Besides the aforementioned pro-tumor functions, TGF-β signaling exhibits suppressive effects in cancer (Dong et al. 2007; Guasch et al. 2007). While the expression of type III TGF-β receptor (TbRIII) decreases during breast cancer progression, restoring TbRIII expression suppresses tumorigenicity (Dong et al. 2007).

The Pro-tumor Activity of MSCs

The pleiotropic effects of MSCs that promote tissue repair and regeneration may also confer pro-tumor functions to these cells. For instance, metastatic human breast carcinoma cells were found to induce the secretion of the chemokine (C-C motif) ligand 5 (CCL5) from MSCs, which enhanced tumor invasion (Karnoub et al. 2007). Seminal reports demonstrated that MSCs could also inhibit apoptosis in tumor cells by secreting pro-survival factors such as VEGF and bFGF (König et al. 1997; Dias et al. 2002). Numerous studies converged on the finding that MSCs contribute to cancer pathogenesis by releasing inflammatory factors that promote immunosuppressive effects. For example, an in vitro study showed that MSCs isolated from gastric tumors mediate cancer progression through the secretion of IL-8 (Li et al. 2015). This pro-inflammatory chemokine favors the recruitment of leukocytes. It is known that recruited leukocytes, such as macrophages and neutrophils, facilitate cancer initiation and progression (Guo et al. 2017; Powell et al. 2018). Similarly, MSCs are able to secrete TGF-β that promotes macrophage infiltration at the tumor site and facilitates tumor escape from immune surveillance (Kim et al. 2006; Byrne et al. 2008). Compelling evidence indicates that MSCs can also support tumor angiogenesis, an essential process in cancer progression that supplies tumors with oxygen and nutrients. For instance, MSCs recruited in breast and prostate tumors were found to increase the expression of angiogenic factors, including TGF-β, VEGF, and IL-6, which contribute to tumor growth and vascularization (Zhang et al. 2013).

Similarly, a correlation between increased expression of TGF-β and higher microvessel density was observed in hepatocellular carcinomas of mice receiving intravenous injections of human MSCs (Li et al. 2016), which further supports that MSCs may enhance tumor angiogenesis via TGF-β. Furthermore, MSCs can also respond to soluble factors secreted from cancer cells and differentiate into CAFs, a cell type within the tumor microenvironment capable of promoting tumorigenesis (Mishra et al. 2008). In particular, TGF-β secreted from cancer cells plays a critical

role in the differentiation of MSCs into CAFs (Jotzu et al. 2011; Barcellos-de-Souza et al. 2016; Aoto et al. 2018).

It is known that the transition of MSCs into CAFs contributes to tumor progression in part by their active secretome. Profiling of the secretome shows that it is rich in many bioactive molecules, including immune-modulating agents (CXCL12, granulocyte-macrophage colony-stimulating factor), pro-angiogenic factors (VEGF, TGF-β, PDGF), pro-survival factors (hepatocyte growth factor, insulin-like growth factor 1, interleukin 6), and extracellular matrix modulators (MMP, tissue inhibitor of metalloproteinases) among others (Kalluri 2016). Cell engulfment has also been identified as an interacting process between MSCs and cancer cells that enhances tumor aggressiveness. A recent report demonstrated that breast cancer cell engulfment of MSCs leads to changes in the transcriptome profile of tumor cells. These changes are mainly associated with oncogenic pathways. This MSC engulfment enhances epithelial-to-mesenchymal transition, stemness, invasion, and metastasis of breast cancer (Chen et al. 2019).

The Imprint of Disease on MSCs

One of the strategies to obtain MSCs for therapeutic purposes is an autologous approach. These cells are collected from patients by more or less invasive methods, isolated, seeded in culture under good manufacturing practice (GMP) quality standards, and re-injected into the patient. Nevertheless, when body's repair mechanisms are insufficient or ineffective, this treatment results in a homeostatic imbalance in the organism, producing degradation and disease and compromising the pool of endogenous cells, thus resulting in low efficacy. Some conditions/diseases provoke changes in the BM microenvironment, which is one of the primary sources of MSCs, thus producing changes in the endogenous pool of MSCs and altering their biological features (Mazzanti et al. 2008). MSCs from patients with acute myeloid leukemia showed abnormal biological properties, including morphological heterogeneity, limited proliferation capacity, and impaired differentiation and hematopoiesis supportability (Zhao et al. 2007).

MSCs derived from patients with multiple myeloma showed impaired immune-inhibitory effects on T cells, decreasing their osteogenic potential (Li et al. 2010). Poor proliferation, differentiation potential, and cytokine release defect were found in BM-derived MSCs derived from patients with aplastic anemia, another hematopoietic disorder (Chao et al. 2010; Bacigalupo et al. 2005). Although the mechanisms remain unknown, MSCs appear to be involved in autoimmune pathologies. For instance, MSCs derived from patients with autoimmune diseases display the following altered functions; MSCs from rheumatoid arthritis (RA) patients have an impaired ability to support hematopoiesis and lower proliferative and clonogenic potentials (Papadaki et al. 2002; Kastrinaki et al. 2008). MSCs from immune thrombocytopenic purpura (ITP) patients have a reduced proliferative capacity and a lower inhibitory effect on T-cell proliferation than MSCs from healthy donors (Pérez-Simón et al. 2009). MSCs from systemic lupus erythematous (SLE) patients

display deficient growth, abnormal morphology, and upregulated telomerase activity (Nie et al. 2010; Sun et al. 2007). MSCs from systemic sclerosis (SSc) patients display early senescence (Cipriani et al. 2007). In metabolic diseases such as diabetes, alterations in autologous MSCs have also been documented.

A study using MSCs from type 2 diabetic mice showed that the number of these cells was diminished and their proliferation and survival abilities were impaired in vitro. Moreover, diabetic MSC engraftment produced only limited improvement in the diabetic subjects and could not produce the same therapeutic outcomes as in their nondiabetic counterparts in vivo (Shin and Peterson 2012). Advanced glycation end products (AGEs) accumulate in the tissues of aged people, and these products are involved in diabetes and musculoskeletal diseases. In 2005, Kume and colleagues (Kume et al. 2005) investigated the effect of AGEs on MSCs. They showed that AGEs inhibited MSC proliferation, induced MSC apoptosis, and interfered with MSCs' differentiation into adipose tissue, cartilage, and bone. Type 2 diabetes-derived Ad-MSCs have been found to have functional impairments in their multilineage potential and proliferative capacity because of prolonged exposure to high glucose concentrations (Cramer et al. 2010).

Diabetic-derived Ad-MSCs have an altered phenotype related to plasminogen activator inhibitor-1 (PAI-1) expression levels and display reduced fibrinolytic activity (Acosta et al. 2013), which suggests that the immunogenicity of MSCs could have associated effects on the coagulation system (Wang et al. 2012; Moll et al. 2012). Thus, MSC-based therapy could lead to thrombotic events in particular recipients. Although the possibility of healing with autologous cells is desirable, little is known regarding the influence of different disease states and concomitant medications on MSCs (Benvenuti et al. 2007; Lee et al. 2009). Thus, although the use of autologous MSCs for cell therapy is widespread, their use in humans must be handled with extreme caution. Researching and analyzing both the risks and benefits of this therapy in individual patients and for each disease are necessary.

Considerations for Clinical Applications

Several clinical trials are in progress to ensure the safety and efficacy of MSCs used as medicaments. For cell-based products, it must be considered that cells are living products and that their interactions with body fluids remain unclear (Acosta et al. 2013; Moll et al. 2014). Phase 1 clinical trials are the first step in investigating a new drug. They include pharmacokinetic and pharmacodynamic studies in which the patient's safety plays an essential role in the development of medicaments. The primary goal of phase 2 clinical trials is to provide preliminary information regarding the drug efficacy and safety supplement data obtained in phase 1 trials. For efficacy and effectiveness issues, other advanced phases are mandatory. In all cases, one cannot consider these issues (efficacy nor effectiveness) unless phase 3 clinical trials are developed (García-Bernal et al. 2021; Hmadcha et al. 2020; Escacena et al. 2015). Usually, safety evaluations are based on possible complications derived from the procedure in a time-dependent manner after administering the cells. Efficacy

parameters focus on the improvement of clinical effects at a given time. MSC-based cell therapy is a relatively new therapeutic option for certain diseases, and data regarding the long-term monitoring of patients remain lacking.

Nevertheless, the administration of MSCs is considered a feasible and safe procedure with no adverse events reported. However, the risks associated with stem cell therapy (Herberts et al. 2011) must be considered because these risks increase the probability of an adverse event. The cell source, donor origin, product manufacturing, and recipient disease status are important factors related to the safety and efficacy of MSCs. In this regard, the use of bovine proteins in the medium used to culture these cells (Horwitz et al. 2002) and the observed formation of ectopic tissue in animal models (Breitbach et al. 2007; Kunter et al. 2007), as well as malignant transformation (Wang et al. 2005; Røsland et al. 2009) and immune responses, must be evaluated before wider clinical applications and registration are accepted.

Safety Concerns

Cell therapy is incredibly complex due to the nature of the product. The mode of action is not always clear, and the potency tests are imprecise, by which it might not be possible to predict the risks thoroughly. When considering the use of expanded MSCs ex vivo for clinical applications, it is necessary to consider a series of potential risks that could affect the cellular product.

The administration of stem cells could affect the host's immune system. These cells could directly influence the immune system (e.g., pro-inflammatory environment) or have an immunomodulatory effect. Although MSCs have been considered immune-privileged in this regard, long-term exposure to the culture medium can make them more immunogenic by positively regulating the normal set of histocompatibility molecules (Moll et al. 2011, 2014). On the one hand, the allogeneic use of the cells entails a greater risk of rejection by the immune system. This rejection could lead to a loss of the function of the administered cells, and consequently, their therapeutic activity could be compromised. The use of immunosuppressants could limit these risks, but, in turn, could cause adverse reactions due to immunosuppressive medication.

On the other hand, MSCs isolated from healthy donors have shown uniform and consistent properties, while patients with some degenerative and inflammatory disease differ in their biological and functional characteristics (Capilla-González et al. 2018; Rennert et al. 2014). In this regard, studies with MSCs from diabetic patients suggest that the hyperglycemic environment and other metabolic disorders associated with diabetes affect the endogenous cellular reserve and their proliferation, differentiation, and angiogenic capacity, among other cellular characteristics (Minteer et al. 2015; Rennert et al. 2014; George et al. 2018; Moll et al. 2019). Once infused in the recipient, the cells come into direct contact with the tissues, bloodstream, and other host cells; the cell-recipient interaction process still needs a thorough investigation and characterization.

Physiologically, MSCs reside in the perivascular compartment of almost every tissue (Bianco et al. 2008; Crisan et al. 2008); however, one of the hurdles to the sustained success of their therapeutic effect is early cell loss. This is primarily due to the incompatibility responses after systemic infusion of cells, a reaction termed as instant blood-mediated inflammatory reaction (IBMIR) suggesting that the immune and inflammatory system reacts to cells that generally are not in contact with the blood circulation (Gupta et al. 2014; Moll et al. 2011, 2014, 2019, 2020; Bianco et al. 2008; Crisan et al. 2008; Nilsson et al. 2014). Even more, it has been further shown that different MSC products display varying levels of highly pro-coagulant tissue factor, a decrease in tissue plasminogen activator (tPA), or an increase in PAI-1 and may adversely trigger the IBMIR or microthrombosis in the target tissue (Acosta et al. 2013; Moll et al. 2019). Although MSCs are considered to be safe, they can promote fibrinolysis (Hashi et al. 2007; Neuss et al. 2010; Moll et al. 2020).

Safety and efficacy are the basic pillars that support the viability of clinical application to treat any disease. Except for hematopoietic stem cell transplants, stem cell therapies used to treat any disease are considered medicinal products; therefore, their development, approval, and use must be per the specific standards established nationally and internationally for such medicines. Thus, regulatory authorities guarantee the safety of the studies (Fig. 2).

Cell Manufacturing for Clinical Use

Except for hematopoietic stem cell transplants, stem cell therapies used to treat any disease are considered drugs; therefore, their development, approval, and use must be per the specific standards established for such medicines nationally and internationally. In this context, MSCs are now considered as "cellular medicament" and are called advanced therapy medicinal products (ATMPs) and are under regulation No. 1394/2007 (Escacena et al. 2015; Gálvez et al. 2013). Relating production processes and development staff, clinicians and researchers must achieve GMP procedures under European regulations (Sensebé et al. 2013; Gálvez et al. 2014). Currently, no standardized manufacturing platform exists, although most facilities employ standard release criteria to measure sterility, viability, and chromosomal stability to meet European or FDA regulations (Phinney 2012; Iglesias-López et al. 2019).

Although regulation establishes common parameters to follow, different protocols are used to isolate these cells, and the processes, plating densities, and reagents used cause the results to differ from each other. Donor selection in terms of age and disease status is another variable to consider due to known MSC donor-to-donor heterogeneity (Phinney et al. 1999). The cell source is another important factor related to the efficacy of the product. As reported previously, MSCs derived from different tissues do not consistently achieve the same level of efficacy. Additionally, culture media used for the production of MSCs could affect the basic characteristics of cells; thus, designing a fully defined medium free of animal and human origins is crucial.

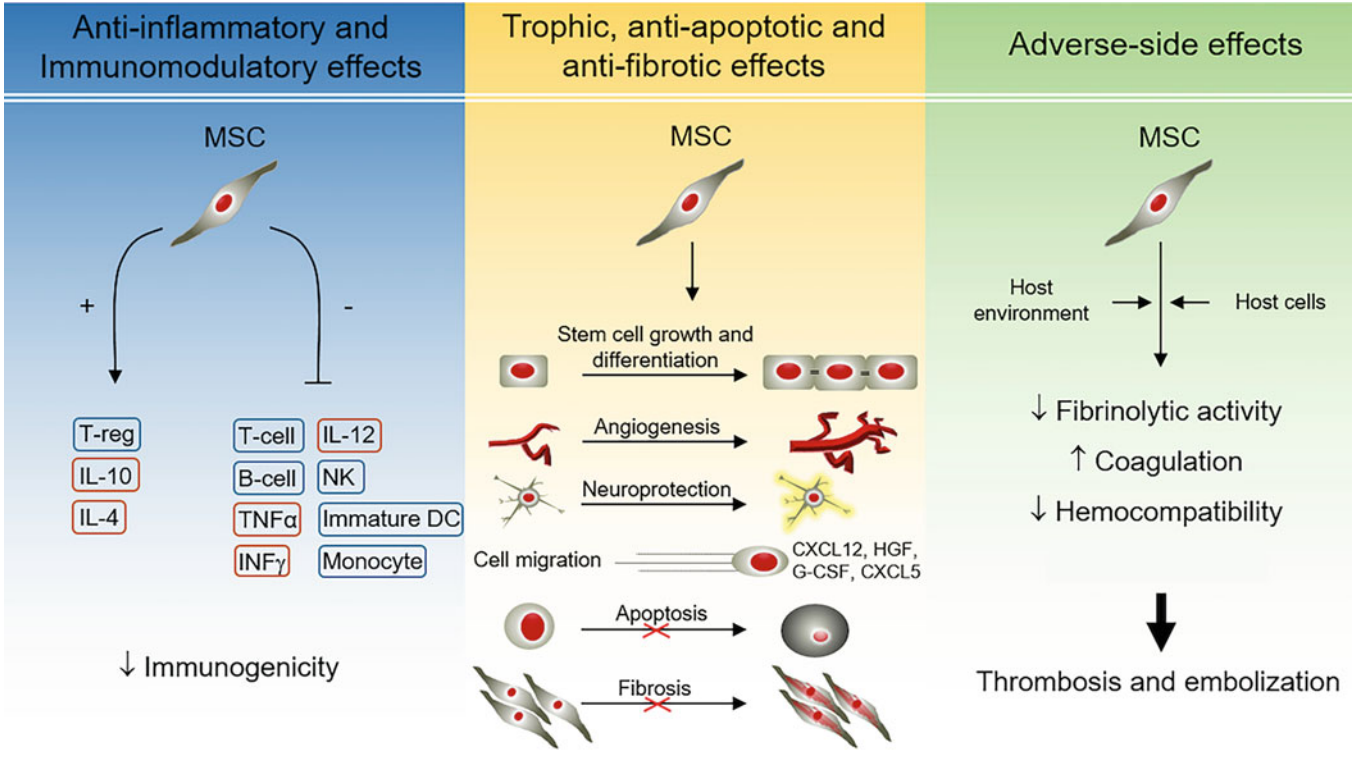

Fig. 2 The MSCs possess a wide range of paracrine effects, including anti-inflammatory, immunomodulatory, trophic, anti-apoptotic, and anti-fibrotic properties. These are mostly mediated by molecules released by MSCs, but also by direct cell-cell contacts. For cell-based therapy, the paracrine properties of MSCs have beneficial effects for the patient. However, the interaction between MSCs and the host can lead to adverse side effects. (Re-published from

Thus far, no MSC-based medicine product has marketing authorization in the European Union, although four gene and cell-based products have a valid marketing authorization awarded by the EMA. However, since 2011, three MSC products have received marketing approval in other regions (Ancans 2012). The MSCs' field continues its upward progression, with a growing number of established companies established and ongoing clinical trials, but remaining challenges must be overcome. Bottlenecks exist regarding donor selection, cell sources, isolation protocols, culture media used, open-culture systems, bioreactors, and recipient disease status. Establishing a standardized and comparable process is also crucial to ensure biological and functional equivalence between product lots.

Considerations for Cellular Medicament

General Considerations

The cell expansion and culture protocol are not standardized, although the regulatory agencies (e.g., EMA, FDA) recommend a set of standards to be followed to produce cellular drugs. Currently, there is no protocol or universal definition for stem cell culture and expansion. The different sources of origin, and the different methodologies for obtaining tissue cells, make it very difficult to compare research groups in search of the fastest, most effective, economical, high-yielding, efficient, and clinical-grade quality method. Cell viability after the infusion is poor; in this regard, it is known that very few cells survive after infusion. Although the in vivo follow-up is ethically and technically complicated, it is necessary to continue investigating this line to understand the intrinsic mechanisms of integrating the infused cells in the concrete microenvironment.

The cellular dose to obtain the desired effects is also unknown. Investigations with HSCs have revealed that the administration of sufficient cells promotes faster cell recovery and reduces hospitalizations (Mohty et al. 2011). Preclinical studies using murine animal models have established a minimum dose of 1×10^6 cells/kg of weight, a quantity necessary to obtain quantifiable but weak benefits (Shabbir et al. 2009; Mastri et al. 2012). The dose for cellular treatment is probably influenced by the patient's body weight and the biodistribution of paracrine factors secreted by MSCs in the human body; however, most clinical trials use a similar cell dose (Tan et al. 2012; Jiang et al. 2011). The doses used have been insufficient in most cases to show clear therapeutic benefits. This fact leads us to design future trials to test different cell doses. Likewise, the frequency of administration is currently unknown.

Fig. 2 (continued) Soria-Juan 2019, article published under CC-BY terms). Abbreviations: B-cell B lymphocyte, CXCL C-X-C motif chemokine ligand, DC dendritic cell, G-CSF granulocyte colony-stimulating factor, HGF hepatocyte growth factor, IL interleukin, INF-γ interferon-γ, MSC mesenchymal stem cells, NK natural killer cells, T-cell T lymphocyte, TGF-α transforming growth factor α, Treg regulatory T cell

The effectiveness of cell therapy is probably related to the number of others applications (Cobellis et al. 2008; Teraa et al. 2015; Molavi et al. 2016), similar to that established with conventionally used medications. The timing and the ideal number of cellular applications are still unknown. Since conventional medicines are depending on the dose, cellular therapy may need to be adjusted accordingly. The most suitable cell type remains a challenge for regenerative medicine. Knowing which cell type is most appropriate for each particular pathology or if a combination of these would be more recommended is another big issue in cell therapy.

The method for the cellular administration continues without giving conclusive results because cell viability must be preserved as much as possible, and compromised tissue is often associated with ischemia, fibrosis and inflammation, which could impair cell survival, therapeutic delivery of stem cells in the distal areas to the damaged tissue appear to offer some advantage. There are no conclusive findings of a more significant benefit within the existing modes of administration, so this is another variable to have into account for future clinical trials. The desired therapeutic effect depends on many factors since mechanism of action of stem cells in tissue regeneration is likely to be multifaceted. Cellular competition can be dictated by the ability of injected cells to migrate, survive, integrate, differentiate, and produce functional paracrine mediators ("cell-cell interactions"). It is known that many diseases (e.g., diabetes, cancer, etc.) affect the phenotypic and therapeutic properties of stem cells. Finally, for the therapy to be effective, the recipient tissue must respond favorably to the injected cells, which would result in the activation of endogenous regeneration mechanisms (Lee 2010). Understanding integration of the exogenous mechanisms (injected cells) with the endogenous (host) will play a decisive role in the future clinical use of adult stem cells (Acosta et al. 2013; Moll et al. 2019).

Attempts to Improve the Therapeutic Outcomes of Cellular Medicament

Advances in the production compliance under good manufacturing practices (GMP) standards of more sophisticated cellular products are now opening up the way for the second generation of cell therapy clinical trials. One of the reasons why unmodified MSCs have not shown the therapeutic efficacy expected in human clinical trials is that, after their systemic infusion (intravenous), these cells become trapped in the vascular filters (fundamentally the liver and lung) and only a small percentage reach the target tissues. Therefore, strategies must be designed that favor migration, nesting, and localization in the inflammatory and/or infectious focus to increase their effectiveness. Biodistribution and long-term follow-up of these cells in animal models show that only a few cells remain after long periods. This will support the idea that most of the effects of MSCs are based on a "hit and run effect."

To increase the concentration of ATMPs in the injured tissue, the CD44 antigen on MSCs' cell membrane by enzymatic fucosylation has been converted into hematopoietic cell E-selectin/L-selectin ligand (HCELL) glycoform (Dimitroff et al. 2001;

Pachón-Peña et al. 2017). This molecular change favored the migration of the MSCs to the inflamed tissues (Sackstein et al. 2008; García-Bernal et al. 2020). This method, called glycosyltransferase-programmed stereo substitution (GPS) of cell surface glycans, has been optimized for its clinical application so that the reagents used (glycosyltransferases and buffers) have been specifically formulated to preserve cell viability and phenotype (García-Bernal et al. 2021). Moreover, this modification not only increases the adhesion of the MSCs to the endothelium, but it also enhances their transmigration through it by activating the alfa4/beta1 integrin in the absence of chemokine stimulation (López-Lucas et al. 2018). Therefore, this modification by fucosylation could improve the efficacy of the treatment with MSCs by increasing the migratory capacity of the cells to the inflamed tissues after being administered systemically (García-Bernal et al. 2020). Other strategies may include expressing CXCR4. These strategies will help to engineer new generation of MSCs for use when both increased migration and targeting and an increased power are required. Expression of the CXCR4 receptor will increase the migration of the MSCs toward the inflammatory focus (Zhu et al. 2021). On the other hand, the co-expression of the anti-inflammatory cytokine IL-10 and/or the anti-infectious cytokine IL-7 will increase the anti-inflammatory effect (IL-10) and even the anti-infective effect (IL-7) (Mao et al. 2017).

Furthermore, the extensive use of fetal bovine serum (FBS) in the MSC-expansion media represents an explicit limitation for the introduction of ATMP at the clinical level. Currently, cell expansion is carried out in culture media supplemented with FBS (Gottipamula et al. 2013). The SFB used must be a clinical-grade (free of animal pathogens). Associated with the growing demand for MSCs, this has led to a series of technical and ethical conditions of production (using a high number of bovine fetuses) and geographic (zones free of prion diseases), which have had an impact on their price (Kinzebach and Bieback 2013; Wessman and Levings 1999). The substitution of FBS by human serum and platelet lysate also represents technical limitations mainly related to the supply of human material and the absence of uniformity of the lots. All these considerations force the development of robust processes of production of MSC in chemically defined culture media free of animal and human components. These media are supplemented with recombinant proteins (albumin, insulin, TGF-β, and bFGF), iron, selenium, and an antioxidant system (2-mercaptoethanol) (Badenes et al. 2016; Jayme and Smith 2000). Although several serum-free media are found in the literature and market (Chase et al. 2010; Ishikawa et al. 2009), there is still no effective means of functioning.

The therapeutic efficacy of MSCs has been further optimized by genetically modifying MSCs to produce trophic cytokines or other beneficial gene products in numerous preclinical models by transfecting MSCs with viral or non-viral vectors (Jiang et al. 2006; Haider et al. 2008; Kim et al. 2012a, b). These MSCs have been successfully modified to express therapeutic peptides and proteins to express therapeutic peptides and proteins in animal models (Zhou et al. 2021). For example, MSCs expressing thioredoxin-1 (Trx1, a potent antioxidant, transcription factor, and growth factor regulator) improved cardiac function in post-myocardial infarction rat models (Suresh et al. 2015). Simultaneous overexpression of Akt and Ang-1 in

BM-derived MSC not only enhanced their reparability of the infarcted myocardium with sustained beneficial effect (Jiang et al. 2006, 2008), but it also led to non-hypoxic stabilization of HIF-1 to enhance their endothelial commitment (Lai et al. 2012a) and increased their proliferation potential via the involvement of miR-143 (Lai et al. 2012b). The MSCs expressing IL-12 showed potent anticancer activity against melanoma, breast cancer, and hepatoma (Gao et al. 2010; Han et al. 2014). In addition, interferon-γ-expressing MSCs inhibited tumor growth in mouse models of neuroblastoma and lung carcinoma (Relation et al. 2018; Seo et al. 2011). Similar to these advances achieved in animal models, several MSC-based therapies are under clinical development.

Both viral and non-viral vectors, however, have some limitations (Kim and Haider 2001). Non-viral vectors exhibit transient gene expression and low transfection efficiency. In contrast, viral transduction is associated with an increased risk of chromosomal instability, insertional mutagenesis, and proto-oncogene activation, despite the inherent high transfection efficiency (Cheng et al. 2019). It has been reported that adverse immune reactions induced by viral transduction impair transgene stability (Mingozzi and High 2013; Wang et al. 2018). Thus, limitations and adverse responses must be assessed when modifying MSCs by transfection. Several studies have sought to use MSCs derived from induced pluripotent stem cells (iPSCs) to obtain better expansion capacity. In fact, therapeutic transgenes could be inserted into iPSC-derived MSCs before MSC derivation. Such a strategy could eliminate insertional mutations and ensure stable expression of transgenes during a prolonged expansion (Zhao et al. 2015). Therefore, MSCs derived from iPSCs may be a renewable source of MSCs for theranostic applications. It is pertinent to mention that BM-derived MSCs have also been successfully reprogrammed to pluripotent status and used for the efficient repair of infarcted myocardium in an experimental animal model (Buccini et al. 2012).

Interestedly, CRISPR-Cas9 technology was used to obtain highly homogeneous MSCs. Genetic modifications of MSCs can be performed with greater efficiency and specificity using CRISPR/Cas9 technology (Gerace et al. 2017). This is faster, cost-efficient, and easier to use compared to alternatives such as transcription activator nucleases (TALENs) and zinc finger nucleases (ZFNs) (Faulkner et al. 2020). CRISPR/Cas9 has been widely employed in the stem cell field, particularly in MSC research, including knock-in, knock-out, gene activation, or gene silencing. In this regard, the application of CRISPR/Cas9 in MSCs has demonstrated its efficacy in treating diseases, such as myocardial infarction (Golchin et al. 2020). Targeting gene knock-in further promoted the differentiation capacity of MSCs and, in turn, improved the insufficiency of functional cells at local sites (Miwa and Era 2018). Genetically modified MSCs have been evaluated in clinical trials, such as the "TREAT-ME-1" clinical trial, an open-label, multicenter, first-in-human phase 1/2 trial, which aimed to evaluate the safety, tolerability, and efficacy of the application of genetically modified autologous MSCs-apceth-101 in patients with advanced gastrointestinal adenocarcinoma (von Einem et al. 2019). Despite promising advances in this field, further research is still needed to obtain solid evidence on the differentiation and regenerative potentials of MSCs in vivo. Undoubtedly, the

next-generation sequencing and genotyping techniques could serve as valuable tools to improve the efficacy of targeting specific cell types for personalized medicine.

Besides, priming MSCs with exogenous small molecules has been found to boost their therapeutic function. Since current MSC manufacturing cannot meet the requirements of clinical trials in terms of production scale, the alternative is to enhance the function of limited cells by priming MSCs. Cell priming, or cell preconditioning, is a commonly used concept in the field of immunology and has been adapted to the stem cell arena (Lu et al. 2010; Haider and Ashraf 2012; Carvalho et al. 2013; Noronha et al. 2019) by ex vivo addition to MSCs of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1α, and IL-1β. More priming approaches are currently being proposed and optimized to improve MSC function, proliferation, survival, and therapeutic efficacy (Afzal et al. 2010; Kim et al. 2012a, b; Lu et al. 2012; Kim et al. 2018; Mead et al. 2020). In this regard and as mentioned before, other approaches are focused on enhancing the therapeutic effects of cell therapy products regulating their biological characteristics (Mangi et al. 2003; Mei et al. 2007; Lee et al. 2012b; Liao et al. 2017).

The beneficial effects of PDGF-BB to restore the defective phenotype of therapeutic MSCs derived from type 2 diabetic patients have been demonstrated. The pretreatment with PDGF-BB potentiates proliferation, migration, and homing of defective MSCs and recovers their impaired fibrinolytic ability. Furthermore, PDGF-BB has been found to exert its beneficial effects through the ERK-SMAD pathway. Therefore, the pretreatment with PDGF-BB represents a suitable strategy to produce more effective MSCs for autologous therapies (Capilla-González et al. 2018).

Concluding Remarks and Future Perspective

Treatments based on the use of human stem cells are novel and promising therapeutic alternatives for some diseases. Currently, the use of living cells as a medicinal product is becoming realistic. Cell therapy should be safe, pure, stable, and efficient. Cell-based products are more complex and depend on the physiological and genetic heterogeneity of the patient. Obtaining as much information as possible with the appropriate and available technology at our disposal is essential for ensuring the safety, reliability, quality, and effectiveness of the manufactured product. MSCs are leading the way into a new era of regenerative medicine, and their multifaceted features make them powerful candidates to become tools to treat several diseases. However, their indiscriminate use has resulted in mixed outcomes in preclinical and clinical studies. While MSCs derived from diverse tissues share some common properties, they markedly differ in terms of their differentiation abilities, growth rates, healing capacity, and gene expression profile.

Similarly, the disease status of donors and recipients is a critical factor to consider when using MSCs as therapeutic agents because factors such as the MSC behavior with body fluids and specific disease environments remain unclear. Available data suggest that some tissue-specific MSCs are more appropriate than others according

to particular pathologies. Although no severe adverse effects related to the application and testing of MSCs in humans have been reported to date, some evidence has indicated that specific patient profiles are not suitable to be treated with these therapies. Thus, multiple bottlenecks for the standardization of therapeutic protocols exist. Future well-designed clinical trials, advanced-phase clinical trials (phase 3/4), and long-term monitoring of patients are crucial for obtaining additional information regarding the therapeutic use of MSCs.

Cross-References

► Mesenchymal Stromal Cells for COVID-19 Critical Care Patients

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Stem Cells in Wound Healing and Scarring

4

Roohi Vinaik and Marc G. Jeschke

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Abstract

Stem cells are cells with the ability for self-renewal and differentiation into a myriad of cellular lineages. Here, we discuss their potential in skin regeneration, focusing on traumatic and nontraumatic healing and scarring. We identify and

R. Vinaik
Sunnybrook Research Institute, Toronto, ON, Canada
e-mail: roohi.vinaik@mail.utoronto.ca

M. G. Jeschke (✉)
Sunnybrook Research Institute, Toronto, ON, Canada

Division of Plastic Surgery, Department of Surgery, University of Toronto, Toronto, ON, Canada

Department of Immunology, University of Toronto, Toronto, ON, Canada

Ross Tilley Burn Centre, Sunnybrook Health Sciences Centre, Toronto, ON, Canada
e-mail: marc.jeschke@sunnybrook.ca

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elaborate on the various types involved, including embryonic stem cells (ESCs) and ESC-like cells, induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs). We discuss the role of iPSCs and MSCs in attenuating inflammation and fibrosis, thus promoting wound closure in models of defective wound healing and reducing both normal and aberrant scarring (i.e., keloids). In particular, we focus on MSCs and fibrotic changes, detailing their inhibitory function in TGFb/Smad signaling, and thus postinjury scar formation. Furthermore, we elaborate on ESCs and ESCs-like populations, discussing applications in normal skin appendage regeneration and recovery of nonhealing wounds, while ESCs-like cells function as a potential source of profibrotic keloid myofibroblasts. Although ESCs-like populations are implicated in scarring, the discussed studies posit that harnessing certain stem cell subpopulations could be an attractive strategy for rapid, scarless wound healing. This has implications in conditions of chronic inflammation and impaired healing and vascularity (e.g., diabetes) as well as traumatic conditions that necessitate rapid skin regeneration, such as burns.

Keywords

Mesenchymal stem cells · Embryonic stem cells · Stem cells · Therapy · Transplantation · Wound

Abbreviations

ADMSCs	Adipose tissue-derived MSCs
ADSCs	Adipose-derived stem cells
AT	Adipose tissue
BMP-2	Bone-morphogenetic protein-2
CM	Conditioned medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Endothelial-to-mesenchymal transition
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
GDF	Growth differentiation factor
HIF-1α	Hypoxia-inducible factor
IFN	Interferon
IL	Interleukins
iPSCs	Human-induced pluripotent stem cells
LIF	Leukemia inhibitor factor
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
NK	Natural killer
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E_2
SSEA	Stage-specific embryonic antigen

TGF-b	Transforming growth factor
TNF-a	Tumor necrosis factor-a
VEGF	Vascular endothelial growth factor

Introduction

The integumentary system serves as a protective barrier against pathogens, dehydration, and fluid loss (Lee et al. 2006). Damage to skin integrity can occur due to nontraumatic (e.g., diabetic ulcers) or traumatic (e.g., burns) conditions and results in a carefully coordinated wound healing process comprised of overlapping phases of cellular migration, proliferation, and extracellular matrix deposition (Eming et al. 2007). However, disruptions or alterations in cellular signaling may culminate in a spectrum of poor wound healing, from chronic nonhealing wounds to excessive scarring (Eming et al. 2007). Either of the outcomes necessitates extensive, costly medical interventions, impairs patients' quality of life, increases trauma, and enhances mortality. Therefore, significant efforts have been devoted to the development of rapid, scarless wound healing therapies.

Regenerative medicine has emerged as an alternative to traditional wound care and focuses on stem cell therapy. Stem cells are an endogenous reservoir of cells that can self-renew and differentiate into a myriad of cell types. Poor wound healing and chronic wounds are correlated with either depletion in resident stem cells or an insufficient response in extensive injuries such as full-thickness burns (Nijnik et al. 2007; Zouboulis et al. 2008; Van Zant and Liang 2003). Therefore, exogenous administration is an attractive potential therapeutic strategy. In this chapter, we discuss the role of stem cells during normal, impaired wound healing, and "overhealing" (i.e., hyperproliferative scars such as keloids and hypertrophic scars). We elaborate on the key endogenous stem cell subtypes involved in skin regeneration, including embryonic, mesenchymal, epithelial, and melanocytic stem cells. Furthermore, we discuss the role of harnessing stem cells in therapy for patients with defective healing or excessive scarring, underscoring the broad application of stem cell therapy in wound-healing aberrancies.

Wound-Healing Process

Normal Wound Healing

Physiologic cutaneous healing consists of an inflammatory, proliferative, and remodeling phase (Fig. 1) (Gonzalez et al. 2016). Wound healing begins with hemostasis, or coagulation and fibrin clot formation. The fibrin clot serves as a scaffold to recruit key mediators involved in healing, including fibroblasts, keratinocytes, endothelial cells, and leukocytes (i.e., neutrophils and monocytes) (Broughton et al. 2006; Witte and Barbul 1997). In particular, activation of macrophages plays a significant role in the inflammatory phase, and macrophages regulate

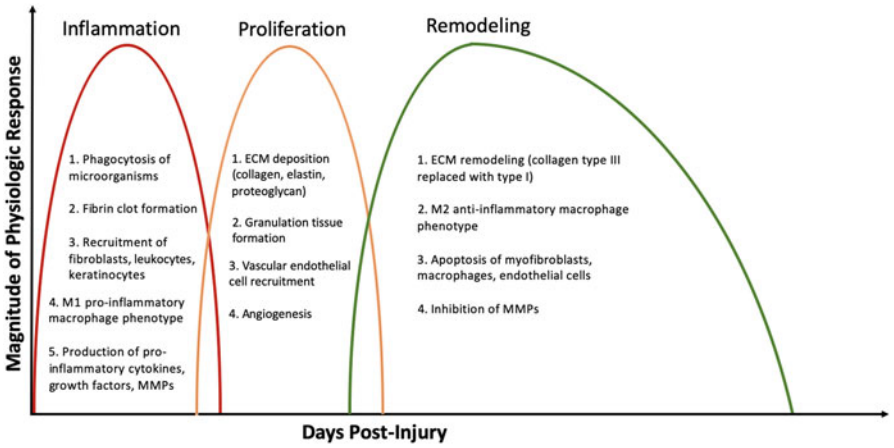

Fig. 1 Stages of post-trauma wound healing

the production of cytokines, angiogenesis, and recruitment of fibroblasts to the wound bed (Rodero and Khosrotehrani 2010). Fibroblast recruitment prompts type III collagen, proteoglycan, and elastin secretion during the proliferative phase, which results in granulation tissue formation (Tracy et al. 2016). This is coupled with the recruitment of vascular endothelial cells by vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF-b) secretion and angiogenesis (Corliss et al. 2016). This phase is followed by a tissue-remodeling phase after wound closure during which macrophages, fibroblasts, and endothelial cells are removed via apoptosis (Larouche et al. 2018). At this point, the wound site contains a primarily acellular collagenous matrix with predominantly type III collagen, which is replaced with type I collagen over time (Xue and Jackson 2015).

In addition to the cells mentioned above, wound healing is in part mediated by stem cells, which secrete paracrine factors to recruit various cells and are present in all skin layers: epidermis, dermis, and hypodermis (Wong et al. 2012; Ito et al. 2005; Levy et al. 2005). In the epidermis, endogenous stem cells are comprised of three key populations – the basal layer of the inter-follicular epidermis, sebaceous glands, and the hair follicle bulge or lower region of the outer root sheath (Fig. 2) (Fuchs 2008). These stem cells have several key features in common, such as expression of K5, K14, and p63 and association with the basement membrane (Fuchs 2008). Under physiological conditions, differentiation of stem cells consistently replenishes the relevant skin components as mentioned above. Interestingly, cells from these components are capable of replenishing each other during injury. For example, reepithelialization in partial thickness injuries relies on the migration of cells from sebaceous glands and the hair follicle bulge (Rittié 2016). While these cells are not essential for wound closure and are eventually replaced with interfollicular epidermal stem cells during recovery, they significantly expedite wound closure (Ito and Cotsarelis 2008). Delayed healing is a risk factor for increased infection,

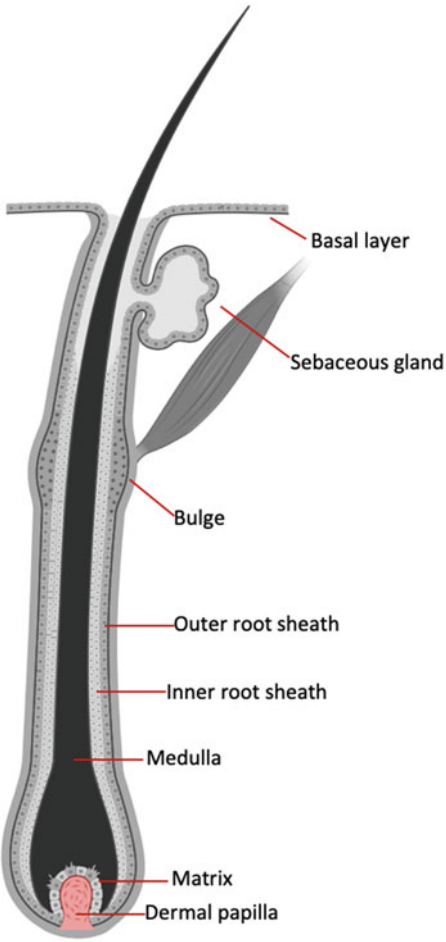

Fig. 2 Anatomy of the hair follicle

hypertrophic scarring, and mortality, rendering these stem cell sources potential targets in regenerative therapy (Finnerty et al. 2016).

Interestingly, dermal signaling (e.g., expression of BMP2 and BMP4) can also contribute to epithelial regeneration (Plikus et al. 2008). An example of this is the contribution of dermal sheath cells to melanocyte maturation. Melanocytic stem cells, which reside in the outer root sheath of the lower permanent portion of hair follicles, are responsible for pigmentation (Lee and Fisher 2014). However, dermal sheath stem cells additionally can differentiate into epidermal melanocytes, likely via mesenchymal-epithelial transition (Li et al. 2011). These cells reside within dermal papillae of hair follicles and are subdivided into three distinct populations based on the transcription factor Sox2. Sox2-expressing cells are involved with Wnt, BMP, and FGF signaling, while Sox2-negative cells exploit Shh, insulin growth factor (IGF), Notch, and integrin signaling (Kellner and Coulombe 2009; Driskell

et al. 2009; Biernaski et al. 2009). Importantly, dermal sheath stem cells can differentiate into a wound-healing fibroblast (myofibroblast) phenotype, which has implications in scarring that will be discussed later (Jahoda and Reynolds 2001). Another dermal stem cells' source is dermal perivascular regions, which function as a niche for mesenchymal stem cells (MSCs) (Jackson et al. 2012). These cells protect the integrity of the local wound-healing matrix via inhibition of matrix metalloproteinase (MMP) pathways and promotion of neovascularization (Jackson et al. 2012). MSCs can also be isolated from various sources, including bone marrow and adipose tissue (AT), and are later recruited to the site of injury in response to factors such as hypoxia-inducible factor (HIF)-1α (Martinez et al. 2017). MSCs, in turn, promote endothelial progenitor cell (EPC) migration, which facilitates angiogenesis and wound closure (Li et al. 2018).

AT is another key source of multipotent skin stem cells (adipose-derived stem cells, ADSCs) that maintain the multilineage differentiation potential (e.g., smooth muscle, endothelium, and bone) (Frese et al. 2016). These ADSCs are associated with perivascular cells and promote vascular stability under normal conditions (Cherubino et al. 2011). Additionally, evidence suggests that ADSCs can enhance macrophage recruitment and anti-inflammatory polarization, fibroblast migration, VEGF-mediated angiogenesis, fibroblast growth factor-2 (FGF2), bone morphogenetic protein-2 (BMP2), and MMPs after injury (Lee et al. 2009). This culminates in neovascularization and deposition, and maintenance of a collagen matrix. While current studies suggest that ADSCs are intimately associated with vascular regeneration, additional research is needed to elucidate their role in the maintenance of skin integrity in normal wound healing (Hutchings et al. 2020; Yu et al. 2018; Lu et al. 2018). Given that AT is enriched in stem cells, ease of isolation, and the plasticity of ADSCs, these cells could potentially be harnessed for wound-healing applications in conditions of impaired healing.

Impaired Wound Healing

Cutaneous wounds are of two subtypes: acute wounds (e.g., lacerations, abrasions, and surgical wounds), which generally heal uneventfully, and persistent, chronic wounds (e.g., diabetic ulcers). The latter occur secondary to interruptions in the healing process as a result of underlying medical conditions, including metabolic diseases (diabetes, obesity) and compromised blood supply (vascular disease, radiation injury) (Ojeh et al. 2015). Impaired circulation and enhanced tissue hypoxemia compromise wound healing by decreasing nutrient and oxygen supply, and hence the metabolic activity of cells involved in the repair, including keratinocytes and fibroblasts (MacKay 2003; Gottrup 2004). Additionally, patients with chronic wounds show an inadequate cellular response to relative hypoxia at the injury site. Under normal wound-healing conditions, hypoxia results in the activation of HIF-1a and recruitment of MSCs to the wound bed (Hong et al. 2014). As indicated earlier, these cells, in turn, promote EPC migration and neovascularization to enhance oxygen delivery to the site of injury (Li et al. 2018). However, EPCs obtained from patients with chronic wounds such as

diabetes show decreased hypoxia-induced adhesion, migration, and proliferation (Vasa et al. 2001; Tepper et al. 2002; Loomans et al. 2004). This culminates in impaired angiogenesis and prolonged ischemia, further compromising wound healing (Capla et al. 2007). As discussed in the context of routine wound healing, ASCs also play a key role in improving vascularization and maintaining the collagen matrix. Complementarily, diabetic wounds exhibit functionally impaired ASCs, which results in diminished growth factor production (Cianfarani et al. 2013).

Combined with abnormal cytokine signaling, decreased growth factor production detrimentally impacts wound-healing outcomes (Galkowska et al. 2006). In addition to promoting revascularization, factors such as FGF also contribute to the recruitment and replication of stem cells (i.e., MSCs), further enhancing growth factor production (Rodrigues et al. 2010). Lack of growth factors is associated with impaired recruitment and function of vascular endothelial cells and multiple stem cell lineages. The source of these factors is macrophages, and the altered macrophage activity (e.g., in diabetic ulcers) is a responsible cause and result of altered signaling pathways (Maruyuma et al. 2007). Macrophages obtained from chronic diabetic wounds release fewer growth factors relative to healthy tissue macrophages, functioning as a significant cause of decreased angiogenesis and impaired healing in these patients (Toma et al. 2005; Yamanishi et al. 2012). Taken together, growth factors and stem cells have a complementary relationship, and aberrancies in either can result in impaired or excessive healing.

Excessive Wound Healing

On the opposite spectrum of healing is overhealing or pathological scarring, which includes hypertrophic scars and keloids. The scarring has significant detrimental physical, social, and psychological consequences, providing an impetus for further research regarding tissue regeneration. Many studies focus on differentiating mechanisms underlying adult versus fetal wound healing, which is scar-free. Interestingly, midgestational fetal wounds heal rapidly and are characterized by regeneration of all skin components (including skin appendages) and maintenance of identical collagen patterns to uninjured tissue (Hu et al. 2014). This contrasts to adult wounds, which lack skin appendages and heal by a fibroproliferative response characterized by a disorganized collagen network, resulting in scar tissue that vastly differs from intact skin (Fig. 3) (Hu et al. 2014).

We compare mechanisms in the fetal healing process to adult skin repair to understand the causative factors underlying pathological scarring. Fetal wounds differ from adult wounds regarding to inflammatory responses, extracellular matrix (ECM) components, and differential growth factor expression (Mast 1992). For example, fetal wounds exhibit a paucity of immune cells, lower levels of the proinflammatory cytokine IL6 and elevated anti-inflammatory IL10, immediate collagen deposition with a higher Type III to Type 1 collagen ratio, higher VEGF and HIF-1a production, and a lack of profibrotic myofibroblasts compared to postnatal wounds to list a few distinctions (Table 1) (Lo et al. 2012). In addition to

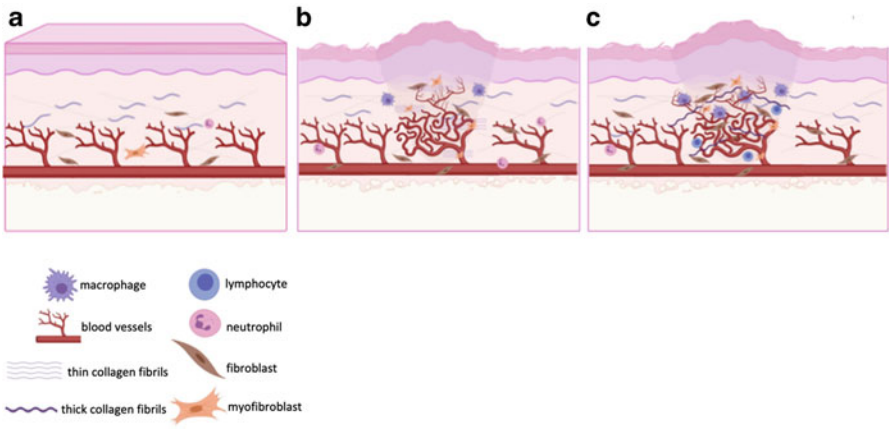

Fig. 3 Anatomy of (**a**) normal skin, (**b**) hypertrophic scar, and (**c**) keloid

Table 1 Comparison between fetal versus adult wound healing. (Modified from Lo et al. (2012))

Characteristics	Fetal	Adult
ECM collagen		
Deposition rate	Immediate	Delayed
Histology	Fine, reticular, and basket-weave	Dense parallel bundles
Type III/type I ratio	Higher	Lower
Cross-linking	Low levels	High levels
Inflammatory response		
Inflammatory cells	Few	Many
IL6 and IL8	Low levels	High levels
IL10	High levels	Low levels
Growth factors		
VEGF	Higher	Lower
TGF-b1 and b2	Low levels	High levels
HIF-1a	Higher	Lower
Fibroblasts and stem cells		
Myofibroblasts (day 14)	Absent	Present
MSCs presence	Higher levels at injury site	Lower levels at injury site

HIF hypoxia-inducible factor-1a, *IL* interleukin, *MSCs* mesenchymal stem cells, *TGF-b* transforming growth factor-b, and *VEGF* vascular endothelial growth factor

the aforementioned factors, fetal wounds also exhibit differences in epidermal and dermal stem cell location and function. For example, postnatal epidermal stem cells reside in the hair follicle bulge, which fetal skin lacks (Nowak et al. 2008). Additionally, as skin development progresses, these epidermal stem cells change in the pattern of cell division. Cell divisions are predominantly symmetric and parallel to the basement membrane initially in embryonic development. In later stages, stem cells begin to undergo asymmetric cell division, which correlates with

a transition from a scar-free to scarring healing phase (Hu et al. 2014). While a significant amount of research is focused on epidermal stem cells, differential responses in dermal stem cells likely contribute to aberrant scarring (Hu et al. 2014). Hereto, we identified stem cells as contributors to skin regeneration and a promising therapeutic option in impaired healing.

However, some studies have provided conflicting results, demonstrating that specific subpopulations of dermal stem cells (MSCs) may increase inflammation and risk of hypertrophic scarring (Zhang et al. 2009a; van der Veer et al. 2009; Ding et al. 2013).

Furthermore, increasing evidence suggests the involvement of stem cells in keloid pathogenesis. For example, aberrant fibroblasts and profibrotic myofibroblasts may originate via epithelial-to-mesenchymal transition from MSCs, which in turn are derived from embryonic stem cells (ESCs) (Lee et al. 2015). In turn, keloid dermis-derived stem cells that express ESCs-specific markers OCT4, NANOG, Sox2, and pSTAT3 (ESC-like cells) promote aberrant fibroblasts and profibrotic myofibroblasts through an MSCs intermediate (Lim et al. 2019; Grant et al. 2016; Bagabir et al. 2012; Zhang et al. 2009b). Likely, establishment of a local inflammatory environment increases the number of keloid-derived precursor stem cells with a distinct MSCs-specific marker expression profile from normal skin precursor cells. The inflammatory niche induces TGFb, epidermal growth factor (EGF), FGF, and insulin-like growth factor (IGF) secretion by keloid-associated stem cells, which in turn results in excessive collagen production and ECM deposition, further recruitment of inflammatory cells and production of inflammatory mediator interleukins (IL) (e.g., IL1a, IL6), and scar formation (Smith et al. 1998; van der Veer et al. 2009). Taken together, studies suggest that the immune system and post-trauma-inflammatory period have an integral role in scarring, which likely occurs via local stem cell dysregulation. In the subsequent section, we will discuss the specifics of the various stem cell populations involved in wound healing.

Stem Cell Subtypes in Skin Regeneration

Stem cells have an integral role in physiologic, impaired, and excessive healing. Given their general prohealing effect, stem cells can putatively be harnessed therapeutically. Here, we discuss the various stem cell subtypes involved in skin regeneration.

Embryonic Stem Sells

ESCs, which are isolated from the inner cell mass of blastocysts, express the cell surface antigens, including stage-specific embryonic antigen (SSEA) SSEA3, SSEA4, T cell receptor alpha locus (TRA) TRA-1-60, and TRA-1-81 and transcription factors OCT4, NANOG, and Sox2 (Thompson et al. 1998; Reubinoff et al. 2000; Clark et al. 2004). The latter transcription factors promote self-renewal genes

while inhibiting differentiation genes, establishing ESCs' pluripotency and the potential to differentiate into any germ layers – endoderm, mesoderm, or ectoderm from which skin develops (Thomas et al. 1998). ESCs can form all somatic tissues and express high levels of telomerase, hence preventing cellular senescence. Depending on the growth factor milieu, ESCs can differentiate into keratinocytes and generate epidermal layers, besides showing proangiogenic properties, thus making them an attractive option for bioengineered skin substitutes (Aberdam 2004; Rufaihah et al. 2010). However, further research is needed before an efficient, controlled expansion and differentiation to particular cell types is feasible. Additionally, studies are required on how to deliver cells in a manner in which they can survive and integrate effectively in patients (de Wert and Mummery 2003).

While these challenges occur in ESCs and adult stem cells, ESCs have additional considerations to adult stem cells. For example, ESCs use is still controversial due to ethical concerns regarding harvesting cells from live embryos (de Wert and Mummery 2003). There are also concerns for potential immune rejection and teratoma formation (Hentze et al. 2009). The latter is likely since ESCs harbor qualities reminiscent of cancerous cells, including self-renewal, persistent proliferation, lack of contact inhibition, and proangiogenic features (Burdon et al. 2002). ESCs also have reduced CpG island methylation in specific genes, allowing for increased gene expression compared to differentiated cells (Altun et al. 2010). This is coupled with hypermethylation and silencing of tumor-suppressor genes, a phenomenon also seen in cancerous cells (Altun et al. 2010). Given that ESCs are potentially tumorigenic, further work is needed before they can be implemented clinically. In the meantime, adult stem cells have garnered greater interest as an alternative source of stem cells for the treatment of diseases.

Induced Pluripotent Stem Cells

While not an endogenous stem cell population, induced pluripotent stem cells (iPSCs) produced from adult-derived cells have significant therapeutic potential and merit an in-depth discussion (Ibrahim et al. 2016). iPSCs are programmed in vitro via induction of Oct4/Sox2/c-Myc/KLF4 or Oct4/Sox2/NANOG/LIN28 (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yu et al. 2007). They are pluripotent cells with the capacity for self-renewal and the ability to differentiate into any adult cell type, similar to ESCs (Ibrahim et al. 2016). As they are derived from adult somatic cells, iPSCs circumvent potential ethical issues faced with ESCs. Furthermore, iPSCs can easily be derived from the adult tissue harvested from multiple sources, including, bone marrow, skeletal muscle, and skin, thus providing a renewable source of pluripotent stem cells (Ahmed et al. 2011a; Buccini et al. 2012; Gorecka et al. 2019). They are also being used for disease modeling (Cagavi et al. 2018).

Due to this ability, iPSCs-derived cells are potentially able to target each phase of wound healing. During the inflammatory phase, they can ameliorate impaired growth factor and cytokine secretion, promoting macrophage, fibroblast, and

keratinocyte secretions lacking in chronic wounds (Casqueiro et al. 2012; Clayton et al. 2018; Kim et al. 2013; Açikgoz et al. 2004). During the proliferative phase, iPSC-derived cells promote angiogenesis and collagen deposition and include endothelial cells, smooth muscle cells, fibroblasts, keratinocytes, etc. (Tepper et al. 2005). Finally, the recruitment of cells such as fibroblasts and myofibroblasts during the proliferative phase impacts the remodeling phase of healing (Grenier et al. 2007).

Interestingly, iPSCs can be induced to produce other stem cell populations such as MSCs, which are multipotent stem cells capable of differentiating into various cell types (discussed in the subsequent section). MSCs obtained from models of poor healing (e.g., diabetic wounds) exhibit impaired proliferation, differentiation, and production of proangiogenic factors (Aasen et al. 2008). IPSCs-derived MSCs may have a better wound-healing potential, and we later discuss iPSCs-based treatment in conditions of poor healing. However, while iPSCs may have a prohealing advantage over MSCs, several potential issues are associated with their implementation. Like ESCs, iPSCs exhibit tumorigenic potential and can form teratomas when undifferentiated (Ahmed et al. 2011b; Gledhill et al. 2015; Krause et al. 2001). Currently, there are strategies to minimize teratoma risk, including differentiation before cell transplantation (Bedel et al. 2017). Another potential issue is that initial production required retroviral transfection with the risk of viral integration into the host genome and insertional mutagenesis. However, more recent techniques are nonintegrative, circumventing potential safety issues (Haridhasapavalan et al. 2019; Malik et al. 2013; Deng et al. 2015). Taken together, iPSCs have a potential application in wound healing, although further studies regarding their safety profile and improvements in iPSCs' generation are needed at this point.

Mesenchymal Stem Cells

MSCs are progenitor cells for connective tissue found in multiple sites, including AT, bone marrow, and nerves (Danisovic et al. 2009). To classify as an MSC, cells should exhibit plastic adherence, express specific cell surface markers (CD73, CD90, and CD105), and lack CD14, CD34, CD45, and HLA-DR, and be able to differentiate in vitro into either adipocytes, chondrocytes, or osteoblasts (Dominici et al. 2006). While the aforementioned characteristics apply to all MSCs, slight variations are depending on the tissue of isolation. Here, we focus on adipose tissue-derived MSCs (ADMSCs), which express the previous factors plus CD29, CD44, CD71, CD13, CD166, and STRO-1 (Ullah et al. 2015).

Akin to ESCs, MSCs have beneficial features for skin regeneration applications, including self-renewal, the ability to home toward wounds, rapid proliferation, and the capacity to differentiate into a myriad of cell types (Sackstein 2004). Their prohealing effects can be attributed in part to the release of growth, cell recruitment, and immunoregulatory factors in response to inflammatory mediators that accumulate at the site of injury (Prockop and Oh 2012). An added advantage over ESCs is that MSCs are not immunologically active due to low MHC1 and lack of MHCII and costimulatory CD80, CD40, and CD86, which protects MSCs from natural killer

(NK) cell lysis (Rasmusson et al. 2003). Furthermore, MSCs can inhibit NK and cytotoxic T-cells via various pathways, such as secretion of human leukocyte antigen G5, leukemia inhibitor factor (LIF), and interferon (IFN) (Selmani et al. 2008; Nasef et al. 2008; Sheng et al. 2008). More specifically, MSCs induce T-cell apoptosis, which enables macrophages to produce TGFb, thus promoting the generation of regulatory T-cells and macrophage phenotype switching to anti-inflammatory subtypes (Ohe et al. 2015; Barrandon and Green 1987; Green 2008). These immunomodulatory effects depend on the quantity and type of cytokines present and diminish the risk of immune rejection, making MSCs a viable option in inflammatory conditions and other clinical applications (Mansilla et al. 2005; Falanga et al. 2007). However, the intensity of inflammation regulates MSC-mediated immunomodulation, necessitating a healthy patient inflammatory status for optimal efficacy (Wang et al. 2014).

Recent research identified ADMSCs within subcutaneous tissue, purporting a skin regeneration role (Marfia et al. 2015). In this context, ADMSCs have several advantages, including ease of harvesting and enhanced proliferative and immunosuppressive properties (Jacobs et al. 2013; Pachón-Peña et al. 2011). The ability for ADMSCs to proliferate, differentiate, and migrate is inherently dependent on the local milieu. ADMSCs home in on the injury site via enhanced expression of CXCR-4, which modifies local immune cell (i.e., macrophages, T-cells, B-cells, and dendritic cells) inflammatory phenotypes to a healing, anti-inflammatory one likely via TGFb or growth differentiation factor (GDF) GDF11-mediated activation (Baharlou et al. 2017; Hyldig et al. 2017; Mazini et al. 2019). Production of TGF-b and GDF11, growth factors (i.e., FGF, VEGF), and cytokines, i.e., IL6, tumor necrosis factor-a (TNF-a), is likely induced by local IL6 production during the inflammatory phase (Mazini et al. 2020). Taken together, ADMSCs interact with dermal fibroblasts via cytokine and growth factor production to regulate their microenvironment. ADMSC secretome stimulates the migration and proliferation of dermal fibroblasts and keratinocytes, besides collagen and elastin deposition (Choi et al. 2018; Ferreira and Gomes 2018). Thus, preconditioning ADMSCs with a particular cytokine and growth factor combinations can enhance therapeutic benefits, which is an essential consideration for clinical applications.

Epithelial (Epidermal) Stem Cells

Studies suggest that the epidermis comprises a basal layer containing 2–7% of stem cells (Potten et al. 1979). Although integrins, which are responsible for attaching the epidermal basal layer to the basement membrane, may be a potential candidate, these stem cells lack specific markers. Evidence suggests that epithelial stem cells, including epidermal interfollicular, sebaceous gland, and hair follicle bulge stem cells, have slightly different characteristics. For example, interfollicular stem cells are less potent than bulge stem cells, possibly suggesting that they are the progeny of bulge cells (Alonso and Fuchs 2003). Interfollicular cells lack a distinct niche and are more easily induced to proliferate, although injuries that destroy the interfollicular

epidermis (i.e., superficial burns) but leave hair follicles intact can regrow epithelium and do not need grafting (Alonso and Fuchs 2003; Green 1991). Hair follicle bulge cells are multipotent cells capable of differentiating into all skin epithelial lineages (e.g., hair follicles, keratinocytes) and forming a stratified epidermis in vitro (Rochat et al. 1994). Taken together, this suggests that epithelial stem cells have different functions and importance, which has implications in skin regeneration and necessitates a greater understanding of the underlying signaling pathways.

In particular, Wnt/beta-catenin signaling is associated with the ability of epithelial cells to exhibit multipotency features (Zhou et al. 1995; Gat et al. 1998; Andl et al. 2002). While only bulge cells are considered multipotent, stabilization of skin beta-catenin induces adult interfollicular epidermal cells to behave similarly to embryonic skin. That is, they can differentiate into epidermal cells or hair follicles. Wnt signaling in embryonic skin epithelial cells results in beta-catenin-mediated activation of the DNA-binding protein family Lef/Tcf (Nusse 1999). When skin MSCs inhibit the BMP pathway, multipotent epithelial stem cells express Lef1 and commit to hair follicle formation (Jamora et al. 2003). The extent of Lef1/Tcf activation is essential in stem cell lineage commitment, and overexpression of Lef1 in skin epithelium results in inappropriate hair follicle formation, while interfering with Lef1/beta-catenin association results in hair follicles adopting a sebaceous cell fate (Zhou et al. 1995; Merrill et al. 2001; Niemann et al. 2001). Interestingly, despite consistently elevated stabilized beta-catenin levels and, hence, epithelial bulge stem cell commitment to hair follicle morphogenesis, the overall size of the stem cell niche does not change (Lowry et al. 2005). This suggests a balance between beta-catenin-mediated self-renewal of bulge stem cells and their efflux from the niche.

Under physiologic conditions, hair follicle and other epidermal cells function as distinct lineage niches. However, injury to the epidermis results in the recruitment of stem cells from different epidermal compartments, allowing for repopulation of cells in other epidermal compartments and expediting wound closure. Given endogenous stem cells' role in wound healing, we subsequently assessed stem cells in defective and excessive healing, highlighting the pros and cons of different stem cell types.

Applications in Defective Wound Healing

Chronic wounds, frequently associated with impaired stem cell function, can be managed with clinical approaches involving stem cell application. Several stem cell subtypes have potential in wound-healing management, including ESCs, EPCs, iPSCs, and MSCs (bone marrow and AD). Initially, ESCs were identified for their ability to differentiate into any of the three primary germ layers and, thus, their potential application in wound healing; however, ESCs can generate tumors and are controversial, as discussed earlier (Kanji and Das 2017). Therefore, focus has been shifted to adult stem cells as alternative treatment options. Here, we discuss current stem cell treatments and their effect on various physiologic events involved in wound healing: inflammation, angiogenesis, and fibrosis.

Endogenous bone marrow-derived EPCs have a crucial role in promoting wound healing via angiogenesis. However, migration to the injury site occurs in response to hypoxic factors, which are impaired in conditions such as diabetes (Tepper et al. 2005; Ceradini et al. 2004). Exogenous stem cell transplant is an alternative strategy to circumvent this issue. A study utilizing diabetic murine models demonstrated that injecting CD34+ EPCs into ischemic wounds enhances vascular density and minimizes healing time (Sivan-Loukianova et al. 2003). Furthermore, injection of CD34+ hematopoietic progenitor cells into human chronic sacral ulcers decreased wound volume by 60% within 3 weeks of administration (Wettstein et al. 2014). Although the sample size was small (three patients), this study provided evidence of efficacy with stem cell treatment and, in this case, no tumorigenicity, paving the way for additional human clinical studies utilizing stem cells.

An alternate source of stem cells is differentiated adult somatic cells, which can be de-differentiated to pluripotent iPSCs. For example, murine fibroblasts or keratinocytes treated with a combination of Oct3/4, Sox2, c-Myc, and Klf4 can revert to iPSCs, which in turn differentiate into tissue from all three germ layers (Takahashi and Yamanaka 2006; Aasen et al. 2008). In addition to the ability to induce pluripotency, directing differentiation into particular cell types is critical. In this regard, iPSCs can be selectively differentiated into fibroblasts and keratinocytes and, as an added advantage, elicit an anti-inflammatory response (Lu et al. 2014). Taken together, these characteristics render iPSCs an attractive option for treating chronic wounds and skin conditions (Zhang et al. 2015a). Indeed, iPSCs-derived cells obtained from patients promote healing and enhance collagen secretion (Gorecka et al. 2019). In murine models, iPSCs-derived MSCs injected into the wound site secrete type-VII collagen at the dermal-epidermal junction and improve epithelialization (Nakayama et al. 2018). These iPSCs can be obtained from donors with preexisting conditions (e.g., diabetes, epidermolysis bullosa), allowing autologous treatment and minimizing rejection (Tolar et al. 2011). This is coupled with a proangiogenic potential, allowing for potential applications in ischemic injury (e.g., myocardial infarction, peripheral arterial disease, and retinopathy). However, further studies regarding the safety-enhancement of iPSCs' reprogramming protocols are needed to assure minimal risk of mutagenesis and teratogenicity (Okita et al. 2007).

Similar to iPSCs, MSCs also lack immunological reactivity and are capable of rapidly proliferating and differentiating into a wide range of cell types (Nakagawa et al. 2005; Krause et al. 2001). MSCs express high levels of VEGF and angiopoietin-1 or may function as pericytes, indicating that prohealing effects are due in part to enhanced angiogenesis and stabilization of blood vessels in normal and impaired healing models (Dai et al. 2007; Shumakov et al. 2003; Kwon et al. 2008). Clinically, MSCs-based therapy has also shown to improve healing by improving angiogenesis and dermal vascularity and thickness, increasing reepithelialization and granulation tissue formation, and, more importantly, modulating the immune response (Badiavas et al. 2003; Vojtassak et al. 2006; Cha and Falanga 2007). Irrespective of the source, MSCs-based cell therapy lowers inflammatory cell numbers and proinflammatory cytokines (e.g., IL1, TNFa) and enhances IL10 at the site of injury in animal wound-healing models (Liu et al. 2014a). Furthermore, MSC-based treatment influences macrophage polarization, promoting an anti-inflammatory M2 macrophage

profile in murine wounds (Zhang et al. 2010). Since macrophage profile and inflammation also regulate fibrosis, the immunomodulatory effect putatively attenuates excessive ECM deposition and abnormal scarring, which will be discussed in-depth in the subsequent section.

Stem Cells in Overscarring

MSCs administration increased the tensile strength of wounds and reduces wound contracture and scarring due to (1) anti-inflammatory effects, (2) paracrine signaling, and (3) modified collagen deposition and ECM remodeling (Hu et al. 2018). Prolonged inflammation can induce fibrosis, and the anti-inflammatory effect of MSCs in part accounts for reduced fibrosis. MSCs upregulate prostaglandin E_2 (PGE2) that inhibits IL2 expression and diminishes T cell proliferation in the wound (Németh et al. 2009; Jarvinen et al. 2008; Djouad et al. 2007). PGE2 also facilitates the transition from T_H1 to T_H2 cells, which corresponds with a reduction in IFN expression and upregulation of IL4 (Zanone et al. 2010; Aggarwal and Pittenger 2005). Decreased IFN relative to IL4 favors anti-inflammatory M2 macrophages, which results in decreased proinflammatory signaling (Stout 2010; Varin and Gordon 2009). This is accompanied by the secretion of various factors that promote tissue regeneration.

MSCs secrete various antifibrotic cytokines and growth factors including hepatocyte growth factor (HGF), FGF2, VEGF, IL10, and adrenomedullin (Li et al. 2008, 2009; Chen et al. 2008; Du et al. 2016). In particular, fibroblasts respond to HGF secretion by downregulating collagen I/III and TGFb1 expression via nuclear exclusion of SMAD3, a transcriptional factor associated with several profibrotic genes (Mou et al. 2009; Schievenbusch et al. 2009; Inagaki et al. 2008). In addition to enhanced HGF and VEGF, TGFb1/b2 neutralization or addition of TGFb3 is also associated with the scar-free repair, and MSCs maintain a higher ratio of TGFb3/TGFb1 protecting against abnormal scar formation (Ono et al. 2004; Shah et al. 1995).

MSCs contribute to collagen type III secretion, and a higher type III: Type I ratio is characteristic of scar-free fetal tissue healing (Longaker et al. 1990; Fathke et al. 2004). In addition to targeting collagen deposition, MSCs-secreted factors, such as HGF, upregulate the matrix metalloproteinases MMP-1, MMP-3, and MMP-13 in fibroblasts, and promoting ECM turnover (Kanemura et al. 2008). Interestingly, MSCs may inhibit profibrotic myofibroblast differentiation via HGF secretion (Shukla et al. 2009; Abe et al. 2001). While myofibroblast differentiation may be needed in normal healing, these cells produce excessive ECM compared to dermal fibroblast and may contribute to scar formation (Bucala et al. 1994). Elevated TGFb1, observed in conditions characterized by a prolonged acute inflammatory response, mediates myofibroblast differentiation and, as a result, contributes to the excessive ECM deposition, the formation of tight collagen bundles, and wound contraction resulting in scarring. Downstream effects of MSCs-mediated inhibition of myofibroblasts yield less scar tissue, as witnessed in oral epithelial healing that is characterized by high HGF levels and lack of significant myofibroblast differentiation (Shannon et al. 2006).

Interestingly, ESC-like populations are implicated in fibrosis in models of aberrant healing (i.e., keloids). While MSCs attenuate inflammation and collagen remodeling, MSC intermediates may differentiate into keloid myofibroblasts (Lee et al. 2015). Keloid dermis contains a population of cells that express elevated ESCs-specific markers OCT4, NANOG, SOX2, and pSTAT3 in perivascular cells within keloid-associated lymphoid tissue, serving as a source of aberrant fibroblasts (Lee et al. 2015). This potentially occurs via the previously mentioned MSC intermediate, generated by the endothelial-to-mesenchymal transition (EMT). Interestingly, while MSCs-based treatment may have an actual clinical application in wound healing and scar prevention, endogenous dermal keloid-associated stem cells contribute to keloid pathogenesis. Thus, further studies regarding the complex signaling pathways involved in wound healing and the contribution of stem cells in the process are needed at this time to further enhance our understanding of the molecular mechanisms involved therein.

Stem Cell Therapy in Pathological Scarring

Several studies demonstrated the use of stem cell-based therapy for hypertrophic scar and keloid treatment. For example, iPSCs-conditioned medium (CM) may suppress hypertrophic fibroblast activation, collagen I, as well as, alpha-smooth muscle actin, a marker for myofibroblasts (Ren et al. 2015). Furthermore, iPSCs-CM-secreted factors block inflammatory cell recruitment, decrease adhesion, and mitigate the contractile ability of dermal fibroblasts (Ren et al. 2015).

In addition to multipotent iPSCs, MSCs also could be harnessed for scar treatment. An example of this is Wharton's jelly MSCs (WJ-MSCs), which exhibit antifibrotic properties, and no teratoma formation or rejection (Gauthaman et al. 2012; Bongso and Fong 2013). In addition to preventing fibrosis, WJ-MSCs enhance healing in immunodeficient mice and diminish scarring in other animal models (Sabapathy et al. 2014; Azari et al. 2011). Bone marrow-derived MSCs similarly have been used to treat hypertrophic scars in rabbit models and murine skin fibrosis, facilitating the formation of uniform, basket-weave collagen organization analogous to normal skin with minimal inflammatory cells (Liu et al. 2014b; Wu et al. 2014). This is combined with a marked decrease in profibrotic TGFb1 and upregulation of MMPs (e.g., MMP-2/9/13) (Wu et al. 2014). Alternatively, injection of ADSCs in a rabbit-hypertrophic scar model led to the normal-appearing scars with a reduced scar elevation index (Zhang et al. 2015b). Likely, ADSCs modify the local host microenvironment via the production of antioxidants, free radical scavengers, and heat shock proteins, promoting normal wound healing (Gimble et al. 2007).

Conclusion

In this chapter, we highlight the mechanisms underlying normal and aberrant post-trauma healing and the role of stem cells in these processes. In particular, we discuss how endogenous stem cells can impact all the phases of wound healing:

inflammatory, proliferative, and remodeling. Currently, the studies suggest that application of stem cells may benefit dermal wound healing by accelerating reepithelialization, improving the tensile strength of new tissue, promote healing in chronic wounds, and minimize excessive scarring. However, further work regarding safety and regulation, clinical translatability, ability to scale up production, and efficient means of stem cell delivery is needed before widespread clinical use.

Cross-References

- ▶ Induced Pluripotent Stem Cells
- ▶ Vascular Functional Recovery and Reparation by Human Endothelial Progenitor Cells

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Mesenchymal Stem Cells

5

From Identification and Characterization to Clinical Applications

Maria Alvarez-Viejo and Khawaja Husnain Haider

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Maria Alvarez-Viejo and Khawaja Husnain Haider contributed equally with all other contributors.

M. Alvarez-Viejo (✉)
Unidad de Terapia Celular y Medicina Regenerativa, Servicio de Hematología y Hemoterapia, Hospital Universitario Central de Asturias HUCA, Instituto de Investigación Biosanitaria del Principado de Asturias, Universidad de Oviedo, Asturias, Spain
e-mail: maria.alvarezv@sespa.es

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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Abstract

Since Friedenstein and co-workers first reported bone marrow-derived mesenchymal stem cells (MSCs) during the early 1970s, several researchers have focused on their biology and in vitro and in vivo characterization in experimental animal models. After their initial isolation from the bone marrow, MSCs have also been isolated from almost every tissue including the adipose tissue, liver, skeletal muscle, amniotic fluid, umbilical cord blood, and dental pulp. Following the minimum criteria recommended by the International Society for Cellular Therapy, human MSCs show preferred plastic adherence in culture, can undergo trilineage differentiation (i.e., adipogenic, chondrogenic, and adipogenic), besides the expression of CD44, CD71, CD90, CD105, and lack of CD34 and CD45 expression. Other important markers include Stro-1, SSEA-4, CD146, and CD271. This identification criterion has gone a long way in comparing the findings from independent research labs involved in MSCs research. MSCs show remarkable immunomodulatory and anti-inflammatory properties besides their ability to undergo transdifferentiation and paracrine activity. These characteristics make them ideal candidates for cell therapy and for this reason; there are numerous clinical trials worldwide that use MSCs to treat various pathologies including myocardial infarction for which they have entered into Phase III trials. They also secrete exosomes as part of their paracrine activity, which has led to the emergence of "cell therapy without cells" approach. Several studies have suggested that exosomes, small extracellular vesicles, derived from MSCs could serve as a novel therapeutic tool in the field of regenerative medicine. This book chapter discusses in-depth the advancements in the field of MSCs-based cell therapy and cell-free therapy using their derivative exosomes with a special focus on the clinical perspective.

Keywords

Bone marrow · Cell-free therapy · Clinical · Exosome · Mesenchymal · MSCs · Regenerative · Transplantation

List of Abbreviations

AMI	Acute myocardial infarction acute myocardial infarction
ASC	Adipose stem cell
BM	Bone Marrow

FAK	Focal adhesion kinase
F-CFU	Fibroblast-colony forming unit
GvHD	Graft-versus-host disease
HSCs	Hematopoietic stem cells
IBD	Inflammatory bowel disease
LNGFR	Low-affinity nerve growth factor receptor
MSCs	Mesenchymal stem cells
NGFR	Nerve growth factor receptor
NTR	Neurotropin receptor
UC	Umbilical cord
UCB	Umbilical cord blood
TNF	Tumor necrosis factor
WJ	Wharton's Jelly
WJ-MSCs	Wharton's jelly derived MSCs

Introduction

Mesenchymal Stem Cells: Fist Description in Bone Marrow

The term mesenchymal stem cell (MSCs) is widely accepted today. The existence of cells in various tissues with the ability to differentiate into diverse cell types is assumed. But, when does this concept arise? We go back to the end of the nineteenth century when Goujon (1869) demonstrated that transplanted bone marrow (BM) in a heterotopic site generated bone and marrow de novo (Goujon 1869). A century later, Tavassoli and Crosby advanced knowledge of the potential of BM with their experiments published in a prestigious scientific journal (Tavassoli and Crosby 1968). These authors observed that autologous transplanted marrow fragments survived in various extramedullary sites in the rat, rabbit, and dog. These pioneering works revealed some cellular elements with proliferation and differentiation capacity in the BM. Based on their work, Friedenstein and collaborators would be the first to suggest a cell type in the BM with the ability for differentiation; it would be a different cell type to hematopoietic progenitors. They demonstrated that BM contains a population of cells with a high proliferative capacity that adhered to plastic and that presented a morphology characteristic fibroblast-like. These authors were also the first to propose the ability of these cells to form colonies from a single cell (the fibroblast-colony forming unit (F-CFU)) (Friedenstein et al. 1970). But it would not be until the beginning of the 1990s when the term MSCs was coined. Caplan proposes this name in analogy with "hematopoietic stem cells (HSCs)" (Caplan 1991). The concept of non-HSCs and their presence in BM was reinforced by Pittenger and co-workers' pioneering data published in 1999 (Pittenger et al. 1999).

The abstract published in a prestigious journal says,

> Human mesenchymal stem cells are thought to be multipotent cells, which are present in adult marrow, that can replicate as undifferentiated cells and that have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma. Cells that have the characteristics of human mesenchymal

> stem cells were isolated from marrow aspirates of volunteer donors. These cells displayed a stable phenotype and remained as a monolayer in vitro. These adult stem cells could be induced to differentiate exclusively into the adipocytic, chondrocytic, or osteocytic lineages. Individual stem cells were identified that, when expanded to colonies, retained their multilineage potential.

This text has already summarized two decades ago what has now been widely accepted as MSCs. Numerous laboratories worldwide became interested in this cell type, and many works began to be published from here onwards. However, there was no defined approach to characterize MSCs due to the lack of specific surface markers. Moreover, they constitute a heterogeneous group of subpopulations and possess a distinct expression of surface proteins and differentiation potential besides showing source-specific transcriptome under a given set of culture conditions (Elahi et al. 2016). Hence, various methods of cell isolation, expansion, and characterization protocols have been reported in the literature to ensure a uniform/homogeneous population of cells.

Their low propensity in the BM, lack of surface markers, and diversity in the methods and protocols of isolation, purification, and expansion protocols rendered it difficult to compare the findings originating from independent research laboratories worldwide. To address this critical issue and standardize MSCs preparations for experimental and clinical usage, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has established standard criteria to fully define and characterize human MSCs preparations (Haider 2018). The minimal criteria include plastic-adherence when maintained in standard culture conditions; they must express specific surface antigens, that is, CD44, CD73, CD90, and CD105 and absence of CD11b, CD14, CD19, CD34, and CD45. Besides, they should undergo trilineage (i.e., adipogenic, chondrogenic, and osteogenic) differentiation in vitro (Dominici et al. 2006). Some additional surface markers expressed by MSCs include, CD9, Cd10, CD13, CD29, CD51, CD54, CD117, CD166, and Stro-1. These criteria facilitated the work of many groups involved in experimental and clinical research with this cell type.

The favorable characteristics of MSCs, availability without any moral and ethical issues, ease of availability, simple isolation and undifferentiated in vitro expansion protocols, multilineage differentiation potential, inherent paracrine activity, and robust nature to carry exogenous genetic material for genetic therapy, etc., have made them attractive candidates for clinical application (Haider and Ashraf 2005).

Bone Marrow-Derived MSCs

Currently, obtaining MSCs from BM is well-established in various laboratories around the world. Usually, spinal aspirates are obtained by aspiration of the iliac crest. Mononuclear cells are isolated from the BM aspirate on a Ficoll-density gradient by centrifuging. After washing twice the cells are re-suspended in culture medium and finally seeded into adequate flasks. Thereafter, the medium must be changed every 2–3 days and wait a time that may vary, but it may be around 2 weeks

to obtain the confluence of the cells. To check the quality of the culture-expanded cells, the three parameters indicated above must be fulfilled – adherence to plastic, presence of specific surface antigens, and the ability to undergo multilineage differentiation (Dominici et al. 2006).

Although BM remains the most used, and well-studied and characterized tissue source of MSCs, obtaining MSCs from BM has many drawbacks such as invasiveness of the process, pain and discomfort for the donor, morbidity, due to high proliferative capacity and resistance to apoptosis (Musiał-Wysocka et al. 2019). Moreover, availability in low cell numbers (only 0.001–0.00001 percent of the total cell population) necessitates in vitro expansion, making them immunogenic and may induce genetic instability and chromosomal abrasions (Haider 2006). This searches for alternative tissues to obtain this cell type with the capacity for self-renewal and differentiation.

Isolation of MSCs from Different Sources

Until recently, MSCs have been isolated from almost every tissue in the body. Some tissue sources and their derivative MSCs have been discussed in the following sections. Our focus will be only on some of these tissue sources for MSCs due to their interest in clinical applications.

Adipose Tissue

Adipose tissue, like BM, is derived from the mesenchyme. It is a connective tissue present in all mammals that serve as simple protection of the viscera. It is currently well-supported by the published data that adipose tissue has an endocrine function. It is responsible for controlling of energy metabolism through the storage of lipids (Scheja and Heeren 2019). This endocrine function is attributed to the adipocytes, which are a rich source of endocrine hormones, that is, adipokines and lipokines released in response to metabolic stresses and physiological cues and have their specific targets in the biological system for action (Booth et al. 2016). Since the publication of the early reports showing the presence of stem cells and their characterization as MSCs by Zuk and colleagues (Zuk et al. 2001), adipose tissue-derived stem cells (ASCs) have been extensively characterized and studied for differentiation capacity (Bunnell et al. 2008; Mizuno et al. 2012).

The protocol for harvesting adipose tissue is accessing a concentrated pool of MSCs in the tissue, simple, minimally invasive, and can be performed under local anesthesia that makes adipose tissue ideal for obtaining cells for clinical use in the cellular therapy context (Vallée et al. 2009). ASCs are isolated either by the suction of adipose tissue (SVF) or from the excised human fat by enzymatic digestion followed by purification (Minteer et al. 2013; Alstrup et al. 2019). The quality attributes of the cell preparation depend upon the isolation and purification technique (enzymatic digestion and nonenzymatic) (Gentile et al. 2019). Similarly, the

harvested cells' yield is significantly determined by whether these methods were used singly or combined (Alstrup et al. 2020). Adipose tissue-derived stromal vascular fraction SVF is an attractive therapeutic product obtained in an operating room using an automated device in 60–90 min with prospective clinical utility and efficacy (Han et al. 2015). It is pertinent to mention that collagen digestion requires more time, technical and procedural know-how of culturing the derived cells, a qualified clean room, and working under good manufacturing practices (GMP). In contrast, while SVF requires a less restrictive regulatory pathway. Studies are underway to ascertain whether the cells obtained without enzymatic digestion are identical to those subjected to this process (Chaput et al. 2016; Kokai and Rubin 2016). Researchers are awaiting advances in nonenzymatic separation due to the advantages that this would bring, including eliminating collagenase digestion with the implications that it entails. A review of literature has concluded that subcutaneous human adipose tissue is an accessible and abundant cell source for clinical applications (Kapur et al. 2015). Numerous studies using both ASC and SVF can be found in the clinical trials.org database in this context.

Umbilical Cord

The umbilical cord (UC) connects the fetus to the placenta. The two arteries and a vein in the UC are wrapped in Wharton's jelly (WJ), a gelatinous connective tissue. The UC has characteristics ideal for procuring stem/progenitor cells as it provides abundant stem cells. Their clinical use would not generate ethical conflicts, they are easily obtainable, display low immunogenicity, and have the potential for use in autologous cell therapy (Moreira et al. 2019). Besides Wharton's jelly, MSCs are also isolated from umbilical cord blood (UCB).

Umbilical Cord Blood

Once considered biological waste, UCB contains different subpopulations of stem cells, a unique feature not shared with peripheral blood. They have been isolated and characterized from fetuses/infants of various ages at 19–40 weeks (Iwatani et al. 2019).

Besides being a source of cells for hematopoietic stem cells (HSCs), they are under extensive investigation and characterization as an alternative source of MSCs to the BM-derived MSCs for use in cell-based therapy (Weiss and Troyer 2006). From the ease of availability and simple collection/processing protocols to a lower rejection rate and a higher rate of acceptance, UCB stem cells are considered at par or even superior to the BM-derived stem cells. Given their low immunogenic nature, they have also been used from the allogenic source, which incidentally helps overcome their limited availability from the autologous source. However, the quality and quantity of UCB-derived cells are influenced by various methodological-related parameters, the greatest being the sample volume

(Vasaghi et al. 2013). Some authors have proposed that UCB-derived MSCs show high morphological and molecular similarities with the BM-derived MSCs, including the lack of hematopoietic surface antigens (Erices et al. 2000). Similar to BM cells, UCB-cells constitute a heterogeneous population of cells. In a recently published study, two distinct subpopulations of MSCs were reported and named as short- and long-living cells based on their growth capacity and colony-forming efficiency (Amati et al. 2017). Besides revealing a differential trilineage differentiation potential, the study's immunophenotyping results showed intense surface marker expression of CD90, CD105, CD44, CD13, and HLA-DRA while lacking in HSCs-specific markers, that is, CD31, CD34, and CD45. The cells were also negative CD271. There are reports about the presence of UCB-derived MSCs co-expressing neuronal markers, which show spontaneous neuronal differentiation (Divya et al. 2012). A recently published study has used a combinatorial expression pattern of CD105, CD90, and CD73 (Mishra et al. 2020). They observed that UCB-derived MSCs double-positive for CD105 +CD90+ were CD45+CD34+ immediately after isolation but started to reduce CD45 and CD34 markers during in vitro expansion. The authors also reported that umbilical cord tissues were a much rich source of MSCs than the UCB.

Molecular profiling for stemness-related markers revealed little difference between MSCs derived from UCB, BM, and adipose tissues (Heo et al. 2016). A direct comparison of UCB-derived MSCs and BM-derived MSCs revealed that the former showed more robust chondrogenic differentiation. Bioinformatic analysis revealed donor-to-donor variations in their inherent genetic expression profile pertaining to the pro-angiogenic gene under hypoxic culture conditions (Kang et al. 2018). The presence or absence of MSCs in UCB has remained controversial for some time. For example, Yu et al. (2004) reported that early fetal blood was rich in MSCs; however, full-term UCB was devoid of these cells (Yu et al. 2004). Alternatively, several authors have proposed that the difficulty of obtaining MSCs from UCB was due to their low propensity, as low as 1–2 clones per 10^8 mononuclear cells of UCB (Martins et al. 2009; Bieback et al. 2004; Kern et al. 2006). These data seriously argue against the possibility of considering the UCB as a resource for acquiring MSCs. However, several authors have successfully differentiated UCB-MSCs in vitro to osteogenic, chondrogenic, neural, and hepatic lineages (Liu et al. 2011; Tio et al. 2010; Zhang et al. 2011). Although well-studied and well-characterized, up until now, UCB is not unanimously accepted as a source of MSCs for routine clinical applications.

Wharton's Jelly

Thomas Wharton was the first to describe WJ in 1656, a gelatinous substance composed of various isoforms of collagen and proteoglycans with the principal function to protect the arteries and veins from compression and torsion. They provide a bidirectional flow, delivering oxygen and nutrients that contribute to the adequate development of the fetus and moreover eliminating the waste and carbon dioxide. Human WJ-derived MSCs are emerging as an efficient and advantageous

source of stem cells for experimental and clinical application. Their comparison with MSCs derived from other compartments of the umbilical cord revealed that they are a better choice of cells for clinical application (Subramanian et al. 2015). They exhibit a high degree of self-renewal capacity and multi-lineage differentiation potential, similar to that of MSCs derived from BM (Kern et al. 2006). The ease of availability and noninvasive collection protocols adds up to these advantages. Phenotypically and genetically, they show close resemblance with the embryonic stem cells, but their use and availability are without ethical or moral issues (Marino et al. 2019). Transcriptome profiling of WJ-derived stem cells revealed very low-level expression of pluripotency markers akin to the embryonic stem cells, that is, Nanog, Sox2, Lin28, and POUF1, thus providing a reason for their safety in terms of teratogenicity (Fong et al. 2011). Besides, they also express genes associated with immunomodulation, apoptosis, and chemotaxis. They also release a plethora of growth factors, cytokines, and extracellular vesicles as part of their paracrine activity contributing to their beneficial effects (Puig-Pijuan et al. 2020). There are two popular protocols for the isolation of WJ-derived MSCs, including the one based on the explant technique and the enzymatic digestion method. The enzymatic digestion protocol offers better results because the cell populations obtained are more uniform and homogeneous (Ding et al. 2015). WJ-derived MSCs are attractive cells due to their capacity for proliferation and other characteristics of MSCs like immunomodulatory properties and because the UC is an easy resource to obtain.

Placenta

The human placenta plays a fundamental and essential role in fetal development, nutrition. The placenta's fetal part originates from the blastocyst, whereas the maternal component (decidua) is derived from the endometrium. It is generally believed that it might be a source of primitive cells with immunomodulatory characteristics that render these cells worthwhile for use in regenerative medicine (Evangelista et al. 2008). Besides other types of cells, various studies have demonstrated that the human term placenta is a rich source of MSCs as it is considered one of the structures developed during the earliest stages of embryogenesis (Battula et al. 2008). Considering the complexity of the structure of the placenta, Parolini and coworkers published a paper to show the origin and define a protocol for the isolation of cells from the placenta. One of the main characteristic features of the placenta is its four regions, including the amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal, and chorionic trophoblastic, each one of which respectively offers the following cell populations: amniotic epithelial cells, amniotic mesenchymal stromal cells, chorionic mesenchymal stromal cells, chorionic trophoblastic stromal cells (Parolini et al. 2008). A direct comparison of MSCs derived from these tissues has revealed that MSCs from fetal tissue had higher expansion potential than those derived from the maternal tissue, but they all had different levels of paracrine activity (Wu et al. 2018a). Soncini et al. achieved the isolation of amniotic and chorionic mesenchymal cells. These MSCs were isolated by a mechanical

separation followed by enzymatic digestion (Soncini et al. 2007). Both show MSCs' characteristics, such as fibroblastic morphology, adherence to plastic, and the capacity to form colonies. Both cell types, when analyzed by flow cytometry, showed phenotypes similar to BM MSCs. Diaz-Prado reported the isolation of amniotic membrane MSCs from human placenta by two different protocols for comparison and successfully isolated and in vitro expanded their derived cells for further characterization; however, the cell yield was much higher by Soncini's protocol (Díaz-Prado et al. 2011). These amniotic MSCs are identified by the expression of CD105, CD90, and CD73, plastic adherence ability, and trilineage differentiation and showed excellent osteointegration and bone regeneration potential post engraftment in a rabbit model (Yin et al. 2019). Despite these encouraging data, the discrepancy in their characterization makes it difficult for their progress to regular use in the clinic (Ghamari et al. 2020).

Characterization MSCs Before Culture

As discussed earlier, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy in 2006 proposed minimal criteria to define human MSCs that included preferential adherence to a plastic surface; specific surface antigen expression, that is, CD105, CD73, and CD90, among others, and a simultaneous lack of expression of hematopoietic and endothelial cell-specific surface antigens, that is, CD45, CD34, CD14 or CD11b and HLA-DR); and multilineage differentiation potential to adopt phenotypes similar to osteoblasts, adipocytes, and chondrocytes. These criteria define the minimal requirements to ascertain the purity of MSCs preparation in culture. However, the in vivo identification of MSCs in their "natural habitat" before ex vivo culture has not been well established by any such criterion. They are generally accepted to have a fibroblastic or pericytic origin (de Souza et al. 2016; Soundararajan and Kannan 2018). It would be exciting to establish an appropriate optimized protocol of cell selection that allows the employment of MSCs before their in vitro expansion. A standard protocol to define pre-culture identification markers would guarantee higher purity of the cell preparations than that obtained with selection based on plastic adherence. Many investigators direct their efforts to find a marker to ensure their selection. In an exciting review, Lv and co-workers (Lv et al. 2014) have in-depth reviewed several molecules proposed as markers to identify MSCs before culturing (Lv et al. 2014). The authors have primarily focused on four markers, that is, Stro-1, CD271, SSEA-4 (stage embryonic antigen-4), and CD146 for consideration.

STRO-1

STRO-1 is a cell membrane 75 kd endothelial antigen expressed on MSCs membrane (Ning et al. 2011). It is highly expressed on MSCs. It moves out from the endoplasmic reticulum in response to a decrease in intracellular calcium (Lv et al. 2014). In 1991,

Simmons and Torok-Storb demonstrated Stro-1 enriched F-CFU from human BM with multipotency (Simmons and Torok-Storb 1991). However, this marker's use as an in vivo MSCs "enricher" did not work with the various tissues tested for its expression. Lv et al. (2014) reviewed that Stro-1 is not universally expressed in all reported types of MSCs. Adipose tissue-derived stem cells highly express STRO-1 in culture when differentiated into endothelial lineage (Ning et al. 2011). Its expression is lost from MSCs when the cells are cultured for a longer time in vitro (Gronthos et al. 2003). Various research groups have attempted to establish a relationship between STRO-1 expressions with the MSCs functionality. STRO-1 expression has been related to the immunosuppressive properties of the MSCs (Francois et al. 2005). STRO-1+ cells could stimulate T-cell proliferation more than STRO-1- MSCs. Similarly, another study has attributed the expression of STRO-1 positivity with the paracrine activity of the cells (Psaltis et al. 2010). Pekozer and colleagues have recently shown that STRO-1 positivity is related to trilineage differentiation potential (except for osteogenesis, which was similar to STRO-1- cells) of the cells besides higher clonogenicity and proliferation (Pekozer et al. 2014). Despite these sporadic data, the exact relationship between STRO-1 expression and MSCs functions has not yet been fully established; its expression is related to the primitive status of MSCs.

CD271

CD271 is one of the most specific and prolific markers for the purification of MSCs from the human BM besides UCB. However, CD217+ MSCs derived from UCB were slow in proliferation compared to the ones derived from the BM (Watson et al. 2013). CD271 is also referred to as the low-affinity nerve growth factor receptor (LNGFR), nerve growth factor receptor (NGFR), or p75NTR (neurotrophin receptor). It has been included as a member of the tumor necrosis factor (TNF) superfamily (Thomson et al. 1988). A review performed by Alvarez Viejo and colleagues concluded that CD271 would not be regarded as a universal marker to identify MSCs before culture in vitro. In the BM or adipose tissue, CD271 could be considered a quite suitable marker to isolate MSCs. However, CD271 is inadequate for MSCs' isolation from other tissues such as UC or UCB. Moreover, in the placenta, contradictory results have been obtained by different groups (Álvarez-Viejo et al. 2015). MSCs isolated based on CD271 are highly immunosuppressive and possess lymphohematopoietic engraftment promoting properties. Kuci and colleagues have reported a functional heterogeneity between CD271+ MSCs subpopulations compared with the plastic adherent MSCs. They have shown that both MSCs populations showed differential proliferation and differentiation potentials besides allosuppression but following different mechanisms (Kuçi et al. 2013). Within their derivative clones, the cells were monopotent, bipotent, and tripotent, while their immunosuppressive properties were not consistent with their proliferation or differentiation capacity. A recently published study has reported that CD271-selected MSCs were less angiogenic than their counterparts isolated based on preferential

plastic adherence, although both cell types could populate the scaffolds equally (Kohli et al. 2019).

SSEA-4

SSEA-4 is an early embryonic glycolipid antigen and one of the panels of reliable markers for identification of the undifferentiated human ESCs and cleavage to blastocyst stage embryos (Wright and Andrews 2009). Gang et al. identified the adult BM-MSCs population using SSEA4 (Gang et al. 2007). Conversely, other authors reported no detection of SSEA-4 expressing cells in the unsorted BM (Tormin et al. 2011; Wagner et al. 2005). Therefore, these data suggest that this marker would not be considered an excellent marker to isolate MSCs before culture. A recent study has isolated SSEA-4 expressing cell population from WJ that was also positive for CD90, CD105, CD70, Nanog, and Sox2 (Li et al. 2017). These cells could spontaneously differentiate cells of all three germ layers in vitro. Moreover, they were able to undergo adipogenic and osteogenic differentiation much easier than SSEA4- cells. Maddox and colleagues have reported the presence of stromal cells expressing SSEA-4 in the breast and abdominal adipose tissue and showed the higher potential of osteogenic and adipogenic differentiation potential (Maddox et al. 2012). It is pertinent to mention that breast adipose tissue contained only 0.48% SSEA-4 positive cells sub-population against the 12% propensity of their counterparts in abdominal fat tissue. It is also expressed by some of the cancer cells identifying their malignant character and resistance to chemotherapy (Aloia et al. 2015; Nakamura et al. 2019). Given its association with disease progression in cancers, SSEA-4 expression results in the loosening of the cell-to-cell interaction, loss of epithelial phenotype, and adoption of the mesenchymal phenotype. These cellular and molecular changes are important for the cells to attain migratory capacity, thus contributing to their metastasis (Sivasubramaniyan et al. 2015).

CD146

CD146, also known as Mel-CAM, MUC18, A32 antigen, is a 113 kDa melanoma cell adhesion molecule (CAM) (Wang and Yan 2013). This is primarily expressed at the intercellular junction of endothelial cells. A more recently published study has reported that CD146 is more than merely an adhesion molecule to serve as a receptor for various signaling molecules, thus participating in diverse physiological and pathological processes encompassing angiogenesis to lymphogenesis (Wang et al. 2020c). The soluble form of CD146 derived from endothelial cells is being considered a reliable marker of neuroinflammatory disease (Wang et al. 2020a). Moreover, it mediates FAK activation to help melanoma cells to traverse vessel walls during metastasis in response to VEGF signaling (Jouve et al. 2015). Harkness et al. (2016) published a study showing that CD146 defines a subpopulation of human MSCs capable of bone formation and in vivo trans-endothelial migration. They also

showed that the CD146 sub-population of the BM cells was ideal for use in clinical protocols of bone tissue regeneration (Harkness et al. 2016). The expression of CD146 was found in human MSCs and other sources of MSCs such as adipose tissue, placenta, or dermis, among others (Lv et al. 2014). CD146 expression on MSCs has been attributed to their better therapeutic potential than the cells lacking in CD146 expression (Wu et al. 2016). It is also considered a surrogate marker for MSCs cultured in vitro to predict their differentiation potential. MSCs in the confluent cultures downregulate CD146 expression with concomitant decrease in differentiation potential. Therefore, a moderate rate of medium addition during MSCs culture may ensure their confluence good enough to maintain their differentiation capacity (Jones et al. 2018).

MSCs in the Clinical Perspective

Given their remarkable reparability, immunomodulatory, and anti-inflammatory properties generally attributed to their paracrine behavior and differentiation potential, MSCs have entered into advanced phases of clinical trials for the treatment of various diseases. As discussed earlier, their near ideal characteristics, as discussed in the previous sections, make them best candidates for cell-based therapy. Currently, they are part of numerous clinical trials worldwide that use MSCs to treat various pathologies some of which will be discussed in the following sections of the chapter (Table 1).

MSCs-Based Therapy for Cardiovascular Pathologies

Since the pioneering work of Hamano et al. in a group of five patients in 2001 that the BM cell transplantation for myocardial repair is safe (Hamano et al. 2001), they have been extensively studied in both the small and large experimental animal models of myocardial infarction and heart failure for the repair and regeneration of the injured myocardium (Haider et al. 2008a; Kim et al. 2012; Van der Spoel et al. 2015; Cai et al. 2016). There is mounting evidence in the literature that transplantation of MSCs (both native, genetically modified, and physiologically or pharmacologically preconditioned) in the experimentally injured heart results in decreased infarction size, reduced area of fibrosis, attenuated remodeling, and preserved global heart function (Haider et al. 2008a, b, 2010, 2012; Afzal et al. 2010; Lai et al. 2012; Kim et al. 2012; Haider and Aziz 2017). MSCs have also combined with other cell types to enhance their survival and differentiation potential (Hosseini et al. 2018). BM-derive MSCs have also been reprogrammed to achieve pluripotency status and their derived cells have been successfully using for myocardial repair in experimental animal models (Buccini et al. 2012). These therapeutic benefits have been attributed to a multifactorial mechanism wherein cardiogenesis and vasculogenesis due to cardiac differentiation and paracrine activity of the transplanted cells. Based

Table 1 Summary of the studies involving MSCs from different tissue sources for various disease conditions

Disease	MSCs source	Results	References
Acute graft-versus-host disease	BM-MSC	BM-MSCs was considered second-line treatment for GvHD	Le Blanc et al. 2004, 2008
Inflammatory bowel disease: Perianal Crohn's disease	ASC	Results phase III clinical trial, improved results as compared with placebo, after 1 year of follow-up	Panés et al. 2018
Crohn's Disease	UC-MSC	UC-MSCs were effective in the treatment of Crohn's Disease and produced mild side effects	Zhang et al. 2018
Ulcerative colitis	BM-MSC	Studied the efficacy and safety of BM-MSCs for patients with ulcerative colitis. Two-year follow-up showed immunomodulatory effects and reduction in inflammation and decreased risk of recurrence	Lazebnik et al. 2010
Knee osteoarthritis	ASC	Evaluated the efficacy of autologous ASCs therapy on pain, function, and disease modification in knee osteoarthritis. Results showed that the therapy was safe and effective	Freitag et al. 2019
Knee osteoarthritis	ASC	Satisfactory functional improvement and pain relief for patients that received this ASCs treatment	Lee et al. 2019
Knee osteoarthritis	BM-MSC	Compared the results of MSC with hyaluronic acid, after a year better results were observed with MSCs	Kim et al. 2020
Multiple Sclerosis	BM-MSC	Observed that transplantation of BM-MSCs in patients with multiple sclerosis is a clinically feasible and relatively safe procedure and induces immediate immunomodulatory effect	Karussis et al. 2010
Multiple Sclerosis	BM-MSC	The administration of the cells was intrathecal and the results in some of the patients were encouraging enough for Phase II	Harris et al. 2018
Rheumatoid arthritis	BM-MSC	Suitable safety profile of autologous MSCs therapy in rheumatoid arthritis patients with promising trend for clinical efficacy	Ra et al. 2011
Rheumatoid arthritis	ASC	Observed that the intravenous dose of ASCs in patients was well-tolerated, recommended it appropriate to carry out one more phase of this clinical trial	Álvaro-Gracia et al. 2017
Rheumatoid arthritis	UC-MSC	Authors indicated that the intravenous infusion of was safe	Park et al. 2018

(continued)

Table 1 (continued)

Disease	MSCs source	Results	References
Heart failure	BM-MSC	Intramyocardial injection of autologous MSCs into akinetic non-revascularized segments produced comprehensive regional functional restitution, which in turn improved global LV function	Karantalis et al. 2014
Heart failure	BM-MSC	Successfully reported the safety and feasibility of allogenic MSCs in patients with dilated cardiomyopathy	Hare et al. 2017
Heart failure	BM-MSC	The intracoronary infusion of human BM-derived MSCs at 1 month was tolerable and safe with modest improvement in LVE	Lee et al. 2014
Heart failure	BM-MSC	Intracoronary BMC therapy improved ventricular performance, quality of life, and survival in patients with heart failure	
Heart failure	UC-MSC	Assessed the safety and efficacy in patients with chronic stable heart failure and reduced LV-ejection fraction	Bartolucci et al. 2017
Heart failure	ASC	ASC treatment was safe but did not improve exercise capacity compared to placebo	

on these data, and encouraged by the favorable data in Phase-I and Phase-II trials (Karantalis et al. 2014; **PROMETHEUS trial** (**P**rospective **R**andomized Study **O**f **M**esenchymal Stem Cell **The**rapy in Patients **U**ndergoing Cardiac **S**urgery; Clinical trial identifier: NCT00587990), MSCs-based studies have currently advanced to multiple Phase III clinical trials worldwide, that is, **RELIEF trials** (Clinical trials identifier: NCT01652209; **R**andomized, Open lab**E**led, mu**L**ticenter Trial for Safety and Efficacy of Intracoronary Adult Human MSCs AMI).

Allogenic MSCs have also been assessed to overcome the problem of autologous cells from the aging and diseased donors. A phase I/II pilot clinical study **POSEIDON** (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis; Clinical trials Identifier: NCT01087996) was performed to ascertain a dose-range comparison between autologous and allogenic BM-derived MSCs in 31 randomized patients (1:1) between the two cell type treatment with escalating doses (Hare et al. 2012). Other research groups have also reported similar clinical trials to ascertain the use of allogeneic MSCs (Perin et al. 2015; Jansen of Lorkeers et al. 2015). Hare et al. have successfully reported the safety and feasibility of allogenic MSCs in patients with dilated cardiomyopathy (Hare et al. 2017).

Some of the other trials using TENDO I/M delivery of MSCs include **ESTIMATION trial** (Clinical trial identifier: NCT01394432); **CEP-41750** (Clinical trial Identifier: NCT02032004; Allogeneic Mesenchymal Precursor Cells) for the Treatment of Chronic Heart Failure; **CHART-1 trials** (Clinical trial identifier: NCT01768702); and **CHART-2 trial** (Clinical trial identifier: NCT02317458).

A randomized, open-label, multicenter, Phase-II/III **SEED-MSC** (Clinical trial identifier: NCT01392105) pilot trial assessed the safety and efficacy of BM-derived MSCs (Lee et al. 2014). The cells were delivered by I/C infusion at 1 month after successful revascularization of the infarct-related artery in 58 acute myocardial infarction (AMI) patients.

Similarly, Dr. Timothy Henry is leading another Phase-II clinical study **AMICI** to assess the safety of **A**llogeneic **M**esenchymal Precursor Cell **I**nfusion in Myo**C**ardial **I**nfarction (Clinical trial identifier: NCT01781390). The research involves 105 AMI patients and is expected to complete and release its findings very soon. Some other on-going Phase-II/III clinical trials including **RELIEF** (Clinical trial identifier: NCT0165209), **CIRCULATE** (Clinical trials identifier: NCT03404063), **SEED-MSCs** (Clinical trial identifier: NCT01392105; Safety and Efficacy of Intracoronary Adult Human Mesenchymal Stem Cells After Acute Myocardial Infarction), etc. have also opted for I/C delivery of MSCs in patients with AMI.

I/C route of cell delivery has also been used for myocardial cell therapy using cells other than BM-derived MSCs, that is, CSCs (**CAREMI trial**; Clinical trial identifier: NCT02439398), BM-derived CD133+ (**COMPARE-AMI**; ACTRN12609001045202), and BM-derived AC133+ cells (**STAR**; **S**tem cell **T**ransplantation in patients with chronic he**ARt failure**) which is incidentally one of the most extensive clinical trials using I/C route for BM cell delivery. Interesting use of the I/C route has been the delivery of CDCs in children with hypoplastic left heart syndrome (HLHS) after staged surgery (Clinical trial identifier: NCT01273857).

More recently, Bartolucci and colleagues assessed the safety and efficacy of the UC-MSCs in 15 patients with chronic stable heart failure and reduced LV-ejection fraction and compared them with placebo treated patients (Bartolucci et al. 2017). They reported that intravenous infusion of 1 × 106 cells/kg UC-MSCs was safe. Moreover, they also observed significant improvement in LV-function, and quality of life in patients treated with UC-MSCs during 3, 6, and 12 months of follow-up.

Other than BM, MSCs-derived from other human tissues have been used to assess their efficacy in the patients which include ASC (Clinical trial Identifier: NCT01449032; MesenchYmal STROMAL CELL Therapy in Patients With Chronic Myocardial Ischemia; **MyStromalCell Trial**), and UC-MSCs (Clinical trial Identifier: NCT01739777; **RIMECARD Trial** (Randomized Clinical Trial of Intravenous Infusion UC Mesenchymal Stem Cells on Cardiopathy).

MSCs-Based Therapy for Acute Graft-Versus-Host Disease

Graft-versus-host disease (GvHD) is a severe and sometimes life-threatening complication due to interaction between donor-derived immunocompetent T cells and recipient tissue antigens. Acute GvHD reaction severity is graded from I (mild) to IV (very severe) after allogenic HSCs transplantation that maybe even fatal in some cases (Moreno and Cid 2019; Nassereddine et al. 2017). Although GvHD offers diverse therapeutic targets during its progression, the use of MSCs with

immunomodulatory has been extensively studied as a potential therapeutic option. In 2004, substantial research in *The Lancet* was published, which provided a leap forward in the treatment of GvHD using BM-MSCs (Le Blanc et al. 2004). The study successfully exploited the immunomodulatory properties of haploidentical BM MSCs in patients with treatment-resistant grade IV acute GvHD. Since the publication of these data, Le Blanc and colleagues besides many other research groups have been part of various clinical trials which have advanced to Phase III trials, and in some countries, MSCs-based intervention has achieved a status of second-line treatment for GvHD (Le Blanc et al. 2008; Martin et al. 2010; Galipeau 2013; Wu et al. 2013; Szabolcs et al. 2010). These data provide evidence that the use of BM-MSCs for the treatment of GvHD is safe and feasible (Cheung et al. 2020).

On the contrary, the authors of a recently published systematic review based on 12 studies and 13 on-going clinical trials have concluded that the published literature well supports the safety of MSCs-based treatment for GvHD. Still, it could only provide low-quality evidence that MSCs reduce the risk of chronic GvHD (Fisher et al. 2019). The systematic review results also suggested future studies to optimize the therapeutic intervention's protocol. The systematic review findings can also be interpreted that the outcome of the studies and clinical trials is influenced by various factors (Wang et al. 2017). For example, it is now generally perceived that not all MSCs' preparations are equally effective, necessitating the optimization of MSCs isolation and purification protocols, besides optimizing the dose and injection route (Elgaz et al. 2019). The pro-inflammatory immune profile in the gut of the recipient at the time of MSCs treatment is one of the primary determinants of the therapeutic outcome (Gavin et al. 2019).

MSCs-Based Therapy for Crohn's Disease

Inflammatory bowel disease (IBD) includes two chronic inflammatory idiopathies: ulcerative colitis and Crohn's disease that severely affect the quality of life. Crohn's disease is characterized by the presence of perianal fistulas, treatment of which remains a challenge for therapy with the contemporary therapeutic options. The merging of cell-based therapies using MSCs has reasonable success thus far in the clinical trials to treat IBD via systemic and local delivery (perianal Crohn's disease) (Adak et al. 2017). Lazebnik et al. (2010) studied the safety and feasibility of BM-MSCs in patients with ulcerative colitis (Lazebnik et al. 2010). A 2-year follow-up of the patients revealed the significant immunomodulatory potential of MSCs, leading to a reduction in inflammation and a decrease in the risk of recurrence. These data were substantiated by a recently published meta-analysis that supported the use of MSCs for treating ulcerative colitis (Shi et al. 2019). Similarly, a previously published meta-analysis by Dave et al. had suggested a promising role for MSCs in cell-based therapy in IBD patients despite many challenges in their routine clinical use (Dave et al. 2015).

Searching Clinicaltrials.org, 62 clinical trials for Crohn's disease have either been completed or in progress using cell-based therapy, mostly using MSCs (https://clinicaltrials.gov/ct2/results?cond=Crohn+Disease&term=stem+cells&cntry).

Panés et al. (2018) have proposed the allogeneic ASCs to treat complex perianal fistulas in patients with Crohn's Disease (Panés et al. 2018). The authors published a double-blind phase III clinical trial reporting encouraging data compared to the placebo-treated patients during the 1-year follow-up. They concluded that ASCs provided a safe and effective option in closing fistulas. Although its etiology is unclear, most researchers believe that Crohn's disease is associated with autoimmune response. Considering this hypothesis, Zhang et al. proposed UC-MSCs to treat this disease in 82 patients with confirmed Crohn's disease (Zhang et al. 2018). During a randomized controlled clinical trial, the patients received a weekly dose of 1×10^6 cells/kg for 4 weeks. During the 12-month follow-up, they observed UC-MSCs were effective in reducing Crohn's disease index, Harvey-Bradshaw index, and steroid therapy. Only four patients showed mild signs of the ill-effects relevant to the treatment.

Although the use of MSCs has progressed to Phase-III clinical trials, the underlying mechanism remains elusive. A recent study in an experimental mice model of dextran sulfate sodium (DSS)-induced colitis has revealed that ASCs induce an innate immune memory response in the MSCs-treated animals (Lopez-Santalla et al. 2020b). Cell therapy-treated animals, which received MSCs during the acute phase after DDS treatment, showed sustained protection against inflammation when re-challenged after 12 weeks. The authors concluded that MSCs treatment incurs long-term benefits as they change the regulatory to inflammatory macrophage ratio in *lamina propria* of the colon. Future studies are warranted to have a head-to-head comparison of the safety and effectiveness of MSCs from different tissue sources, although their use in individual studies has generated encouraging data.

MSCs-Based Therapy for Multiple Sclerosis

Multiple sclerosis is an autoimmune disease involving the appearance of focal inflammatory lesions in the substance white matter of the brain characterized by demyelination of the nerve fibers. Karussis et al. 2010 observed that transplantation of BM-derived MSCs in patients with multiple sclerosis is a clinically feasible and relatively safe procedure and induces immediate immunomodulatory effects (Karussis et al. 2010). MSCs also release biologically active secretome due to their paracrine activity that adds to the therapeutic benefits (Gugliandolo et al. 2020). On the same note, MSCs from tissue sources other than BM have also shown encouraging results (Giacoppo et al. 2017; Riordan et al. 2018). These results have been corroborated among many research groups as reviewed by Lotfy et al. (2020).

In 2018, a study was published showing the results from a Phase I clinical trial (ClinicalTrials ID: NCT01933802) that used MSCs-derived neural progenitor cells to treat multiple sclerosis (Harris et al. 2018). The cells' administration was via intrathecal injection, and the results in some of the patients were encouraging enough to propose the execution of Phase II. The safety and efficacy of autologous MSCs to treat multiple sclerosis were further assessed in a Phase I/II clinical study

MEsenchymal StEm cells for Multiple Sclerosis (MESEMS) (Uccelli et al. 2019). MESEMS study has been designed to merge partially independent clinical trials, following harmonized protocols and sharing some critical centralized procedures, including data collection and analyses. With this model, where various clinical trials are grouped, the authors suggest that the results will provide patients and the scientific community with data on the safety and efficacy of MSC for multiple sclerosis. Although not assessed against all forms of multiple sclerosis, treatment with MSCs is a future hope for the patients.

MSCs-Based Therapy for Rheumatoid Arthritis and Osteoarthritis

Encouraged by the experimental animal data that revealed the safety and effectiveness of cell-based therapy for rheumatoid arthritis, the cell-based therapy approach has progressed to the clinical phase of assessment. A direct comparison of MSCs from various sources, including BM, UC, and human deciduous tooth revealed superior outcomes from UC-derived MSCs (Zhang et al. 2019). A recently published meta-analysis of the preclinical studies has demonstrated consistent therapeutic benefits of cell-based therapy in the experimental animal models (Liu et al. 2019b). Although the mechanism of action and beneficial therapeutic outcome is considered multifactorial, it has been reported that the transplanted MSCs control the memory T-cell response (Noymar et al. 2019).

The first pilot clinical study with MSCs therapy autoimmune disorders including rheumatoid arthritis was conducted in 2010 by the Stem Cell Research Centre in Korea. The results were published in almost a decade later (Ra et al. 2011). This study involved the use of ASC. It was considered the first proof-of-concept clinical study that has reported a suitable safety profile of autologous MSCs therapy in 10 patients with various autoimmune disorders, including rheumatoid arthritis patients with promising clinical outcomes' efficacy. Tested in Balb/c nude mice for tumorigenicity even at larger doses, the cell therapy was safe in the patients. Among several studies published in this line, Álvaro-Gracia et al. evaluated the safety and tolerability of the intravenous administration of allogenic ASC in patients with refractory rheumatoid arthritis as a part of Phase Ib/IIb trials including 53 patients (Álvaro-Gracia et al. 2017). They observed that the intravenous dose of adipose tissue-derived stem cells in these patients was well-tolerated. These results agree with the data published by Park et al. (2018), who reported a phase I, uncontrolled, open clinical trial to treat patients with moderate intensity rheumatoid arthritis using UC- MSCs (Park et al. 2018). The authors noted that the intravenous infusion of UC-derived MSCs showed a similar safety profile to BM-derived MSCs. Lopez-Santalla et al. have recently published a comprehensive review of literature documenting both active and closed clinical trials that have focused on using MSCs in rheumatoid arthritis (Lopez-Santalla et al. 2020a). The authors have concluded that a toxicity-free and adverse effects-free use of MSCs in rheumatoid arthritis patients during all the reported clinical trials conducted. However, insufficient data on efficacy have been obtained from the completed clinical trials, most

likely because a large majority of the rheumatoid arthritis patients enrolled in the studies were refractory to conventional rheumatoid arthritis treatments with a long history of the disease.

It is pertinent to mention that only a few examples of the clinical trials published until today wherein a regeneration of the tissue is necessary to treat autoimmune disorders (Lopez-Santalla et al. 2020a). Moreover, there are still concerns about the use of cells from the autologous tissue sources. For example, it has been reported that autologous MSCs obtained from patients with rheumatoid arthritis were functionally impaired, as was observed by their failure to inhibit Th17 (Sun et al. 2015). These data are significant in optimizing the protocols for future cell therapy clinical trials to harness maximum therapeutic benefits from MSCs-based treatment.

Knee osteoarthritis is one of the most commonly diagnosed forms of arthritis, especially in elderly patients after age 65. The underlying cause is the slow but progressive degeneration of the cartilage that protects the knee joint due to physiological aging. Freitag et al. evaluate the efficacy of ASC-based therapy on pain, function, and disease modification in knee osteoarthritis (Freitag et al. 2019). Upon completing the clinical trial, the authors concluded that the therapy was a safe and effective therapy for knee osteoarthritis. These results were substantiated by Lee et al., who used an intra-articular injection of autologous ASC to treat knee osteoarthritis (Lee et al. 2019). They described satisfactory functional improvement and pain relief for patients that received cell therapy. Given that intra-articular injection of hyaluronic acid is a medical option for knee osteoarthritis, Kim et al. (2020) conducted a comparative study between the MSCs and hyaluronic acid-based treatment (Kim et al. 2020). They observed that the MSCs group showed better results during 1-year post-treatment follow-up than the hyaluronic acid group.

Exosomes-Based Therapeutic Intervention

Despite encouraging data emanating from the clinical studies using the cell-based therapy approach, the underlying mechanism of the therapeutic benefits is only partly understood. In addition to undergoing fusion with the host cells and differentiation to adopt morphofunctional phenotype post engraftment to participate in the regeneration process, the paracrine hypothesis has gained wide-spread acceptance amongst the researchers in the field (Haider and Aziz 2017). These proposed mechanisms are correct and supported by substantial data; however, they are not exclusive (Álvarez-Viejo 2020). It is widely accepted that MSCs produce a plethora of bioactive molecules, including cytokines, growth factors, and extracellular vesicles (EVs) containing specific payload (Altanerova et al. 2017). Almost every cell type, including MSCs, releases exosomes as part of intercellular signaling, and given their specific payload, they could serve as a novel cell-free therapeutic tool in the field of regenerative medicine (Haider and Aramini 2020; Nikfarjam et al. 2020). However, there is little headway made in exosomal use in the clinical perspective as is evident from the literature search which did not fetch any records of clinical trials

at least for the diseases included in our chapter. This limited progress of exosomes from bench to the clinic is attributed to the challenges faced in this regard (Forsberg et al. 2020).

MSCs-Derived Exosome for Therapy "Without Cells"

The term EVs includes microvesicles, nanoparticles, vesicles, apoptotic bodies, and exosomes (Pinheiro et al. 2018). The difference between these terms is the size of the vesicles released. Due to the size-heterogeneity between these particles, it was imperative to develop guidelines for their definition. In this regard, The International Society for Extracellular Vesicles proposed the minimum criteria to characterize extracellular vesicles, which requires *EVs as the generic term for particles naturally released from the cell that is determined by a lipid bilayer and cannot replicate,* i.e*., do not contains a functional nucleus* (Théry et al. 2018). The size of exosomes is 60–100 nm with a diverse protein composition between cells, but they share some proteins, such as CD9, CD63, or CD81, for use as exosome markers (Yamashita et al. 2018). Similar to exosomes derived from other cell types, MSCs-derived exosomes participate in intercellular communication and carry proteins, mRNA, and microRNA (miRNA) for delivery to the target cells to facilitate intercellular signaling (Mendt et al. 2019).

The use of exosomes offers several advantages, such as the aversion of introducing exogenous cells, thus avoidance to bring in mutated or damaged genetic material that could harmfully affect the recipient. They are low in immunogenicity (Elahi et al. 2019), but given their inability to divide or replicate, they are static, thus restricting their therapeutic benefits for time duration until they get eliminated from the biological system post-delivery (Phinney and Pittenger 2017). Currently, there are nearly a hundred clinical trials registered in *Clinicaltrial.gov* fetched by the term exosomes. On the same note, there are nearly a thousand trials that include MSCs. The novelty of exosome-based "cell-free" therapy, however, still require optimization of GMP grade exosome production protocols (Chen et al. 2019), their standard markers, ideal donor cells, standardization of their payload, indicated dose, route of administration, etc., and sufficient preclinical data to support their routine clinical use.

MSCs-Derived Exosomes and Acute Graft-Versus-Host Disease

Given the paracrine hypothesis's wide-spread acceptance that the therapeutic benefits of MSC-based cell therapy are mediated via paracrine release of immunosuppressive and immunomodulating factors, cell-free therapy using MSCs-derived exosomes is now fast-emerging as a treatment option for refractory GvHD in an increasingly standardized way (Zhou et al. 2020). Several preclinical studies have reported that MSCs-derived exosomes could be as effective as MSCs-based cell therapy. For example, using a mice model of acute GvHD (aGvHD), Fujii et al. have

shown that systemic infusion of BM MSCs-derived exosomes prolonged the survival of mice with aGvHD, besides alleviating pathologic damage to aGvHD targeted organs via suppression of CD4+ and CD8+ cells (Fujii et al. 2018). The authors attributed the therapeutic benefits of exosomal treatment to the unique miRNA profile of the MSC-derived exosome preparation used for treatment. Similarly, Wang et al. reported that human UC-MSCs derived exosomes could prevent aGvHD in a mouse model after allogeneic HSCs transplantation (Wang et al. 2016). The authors noted that EVs derived from UC-MSC could prevent life-threatening a GvHD by modulating the immune response. Moreover, they could represent a prophylactic method to prevent aGvHD as well. On the other hand, working with a chronic mouse model of GvHD, Lai et al. studied the efficacy and safety of MSCs-derived exosomes to treat this disease. They suggested that MSCs-derived exosomes could improve survival and ameliorate the pathologic damage of chronic GvHD (Lai et al. 2018). A recently published meta-analysis of four studies involving the use of MSCs-derived exosomes for the treatment and prevention (two studies each) of GvHD showed that exosomes were an effective treatment tool as well as for prophylactic application (Gupta et al. 2020). The authors concluded that the use of MSCs-derived exosomes successfully enhanced survival and attenuated histologic findings of GvHD in all four studies.

Supported by the preclinical data, the cell-free therapy approach has progressed to clinical assessment of MSC-derived exosome to treat the GvHD patients but only very cautiously. There is only one Phase I clinical study registered at ClinicalTrials.gov entitle "Effect of UM-MSCs-derived exosomes on dry eyes in patients with GvHD" (Clinicaltrials.gov Identifier: NTC04213248). The study will enroll 27 patients who will receive UC-MSCs derived exodrops (UM-exo). However, the study results are still awaited. Also, we have only found a letter to the Editor in which their use to treat graft GvHD is described. In one patient, Kordelas et al. used an exosome-enriched fraction processed from collected MSCs supernatants instead of administering the MSCs themselves (Kordelas et al. 2014). The patient was stable for several months of post-exosome application. Although the patient died of pneumonia 7 months after treatment, the authors concluded that BM MSC exosomes could be a potentially new and safe tool to treat therapy-refractory GvHD, and most likely other inflammation-associated diseases. Despite encouraging data, there is slow progress for the use of MSCs-derived exosomes for clinical applications, which may be due to the technical and methodological hindrances involved therein.

MSCs-Derived Exosomes for Inflammatory Bowel Disease

The role of MSCs-derived exosomes for the treatment of IBD in general and Crohn's disease, in particular, has been extensively studied in the experimental animal models (Ocansey et al. 2020). Mao et al. investigated the effects of exosomes derived from UC-MSCs in a model of induced inflammatory bowel disease (Mao et al. 2017). According to their findings, UC-MSCs exosomes could substantially alleviate experimentally induced inflammatory bowel disease in a mice model.

While elucidating the underlying mechanism, IL-10 expression was elevated while pro-inflammatory TNFa, IL-1b, IL-6, IL-7, and iNOS were significantly downregulated in the colonic tissue and spleen of exosome-treated animals. Besides, ubiquitination played a significant role in UC-MSCs derived-exosome treated experimental animals (Wu et al. 2018b). The exosome-treated animals were able to better recover tissue structural integrity. These results agree with those published by Liu et al., who demonstrated that systemic administration of exosomes from human BM-MSCs substantially mitigated colitis in various models of inflammatory bowel disease (Liu et al. 2019a). Mechanistically speaking, the colonic macrophages were pivotal in alleviating the disease process as was observed by abrogation of the beneficial effects of exosome treatment by macrophage depletion. They also agreed with Yang et al., who investigated the potential alleviating effects of BM-MSCs EVs in the colitis model (Yang et al. 2015). The authors concluded that the beneficial effects of exosomes from the BM-MSCs were due to the downregulation of the pro-inflammatory cytokines, inhibition of NF-κBp65 signal transduction pathways, modulation of antioxidant/oxidant balance, and moderation of the occurrence of apoptosis, as the possible underlying mechanism. Despite finding several papers published in the preclinical phase, there are no clinical trials registered in clinicaltrials.gov, although their diagnostic use has been attempted (Larabi et al. 2020). The possible explanation for the lack of exosome-based clinical data is the unavailability of standardized protocols for isolation, large-scale reproducible exosome preparations to their protocols for optimum clinical use (Harrell et al. 2020).

MSCs-Derived Exosomes for Rheumatoid Arthritis and Knee Osteoarthritis

Continuing the success story of MSCs-based cell therapy for rheumatoid arthritis and knee arthritis, Chen et al. investigated the therapeutic effects of MSCs-derived exosomes on joint destruction in rheumatoid arthritis (Chen et al. 2018). After treatment with experimentally induced arthritis in the mice model, the authors showed the safety and feasibility of exosome derived from MSC overexpressing miRNA-150. The authors observed a reduction in joint destruction with concomitant inhibition of synoviocyte hyperplasia and angiogenesis. From the analysis of the results, they conclude exosomes facilitate the direct intracellular transfer of miRNAs between cells. Given the pivotal role of miRNAs and their regulatory network in the pathogenesis of rheumatoid arthritis (Zakeri et al. 2019), many other research groups have adopted a similar approach for their use as biomarkers as well as therapeutic targets (Huang et al. 2019). The recently published study by Zheng et al. substantiates these data (Zheng et al. 2020). They also proposed that exosomes derived from BM-MSCs, and more specifically, BM-MSCs secreted exosomal miR-192-5p could delay the inflammatory response events in rheumatoid arthritis. Meng et al. have used exosomes derived from miRNA-320a-loaded MSCs to regulate fibroblasts-like synoviocytes activity by interacting with CXCl9, which are mechanistically involved in rheumatoid arthritis pathology (Meng and Qiu 2020). Cosenza et al. compared the

anti-inflammatory and immunosuppressive properties of MSCs-derived exosomes and microparticles in a mice model of collagen-induced rheumatoid arthritis (Cosenza et al. 2018). The authors observed that both exosomes and microparticles were equally effective in suppressing the inflammatory response, but exosomes were more efficient in anti-inflammatory activity in vivo.

The role of MSCs-derived exosomes and their mechanism remains an area of intense investigation wherein most researchers have used them for both diagnostic and therapeutic purpose, as summarized by Ni et al. and Mianehsaz et al. in their recently published literature reviews (Ni et al. 2020; Mianehsaz et al. 2019). Tofiño-Vian et al. investigated chondroprotective action of exosomes from adipose tissue-derived MSCs from healthy volunteers in vitro using chondrocytes from arthritic patients (Tofiño-Vian et al. 2018). Treatment with exosomes significantly reduced MMP activity and MMP13 expression besides upregulation of anti-inflammatory IL10 in the treated chondrocytes. Their results support the interest of MSCs-derived EVs to develop new therapeutic approaches in arthritic joint conditions. These in vitro results were reinforced by He and colleagues, who investigated the effect of exosome derived from BM-MSCs on damaged cartilage repair and pain in an experimental animal model of osteoarthritis (He et al. 2020). They used a rat model of osteoarthritis established by injection of sodium iodoacetate. After analyzing these results, the authors concluded that exosomes successfully promoted cartilage repair and extracellular matrix synthesis, as well as relieve knee pain in rats with osteoarthritis. A recently published systematic review analyzing 20 published in vivo and in vitro studies revealed positive findings in reduced inflammation, downregulation of catabolic processes, and increased anabolic studies D'Arrigo et al. 2019). However, for optimal benefits of this approach, it is essential that exosome production protocols and identification of patients who could benefit from this novel approach must be identified.

As with most of the diseases discussed in this chapter, rheumatoid arthritis and knee arthritis treatment with exosomes have not yet progressed to the clinical trials.

MSCs-Derived Exosomes for the Heart

Exosomes from various stem cells, that is, ESCs, iPSCs and their derivative progenitors and cardiomyocytes (Khan et al. 2015; Arslan et al. 2013; Kervadec et al. 2016; El Harane et al. 2018), cardiosphere-derived progenitors (Xiao et al. 2016), and MSCs-derived exosomes (Zhang et al. 2016), have been studied for their reparability of the injured myocardium. Exosomes derived from MSCs have also been used to precondition other cells to enhance their survival post-engraftment and promote angiogenesis and reduce cardiac fibrosis in the experimentally induced cardiac fibrosis with concomitantly improved cardiac function (Zhang et al. 2016). Lai et al. (2010) used purified exosomes from MSCs to treat a mouse model of ischemia-reperfusion injury (Lai et al. 2010). The authors reported a successful reduction in infarct size. These data also strengthened the paracrine hypothesis according to which the cardioprotective effects of stem cell-based therapy were

related to paracrine secretions of stem cells that also included exosomes (Haider and Aziz 2017). These conclusions were later supported by Wang et al., who tested EVs secreted by BM-MSC secreted EVs for their pro-angiogenic activity in the infarcted heart (Wang et al. 2017). They concluded that exosome-derived MSCs are sufficient to improve angiogenesis and exerted therapeutic benefits in the experimental myocardial infarction model. Study of the mechanisms involved in exosome-mediated cytoprotection showed AMPK/Akt signaling in the H2C9 cells treated with MSCs-derived exosomes after subjecting the cells to oxidative stress. On the same note, treatment with UC-MSCs-derived exosome was protective for cardiomyocytes in the infarcted myocardium by transferring miR-19a, targeting SOX6, activating Akt, and inhibiting JNK3/caspase-3 activation (Huang et al. 2020). The exosomal payload's role of miRNAs has been eloquently reviewed for their role in myocardial repair Haider and Aramini (2020). Despite extensive studies in the small animal model and encouraging data from large animal models with clinical relevance, there are still no studies registered for their clinical use (Gallet et al. 2017; de Couto et al. 2017).

Multiple Sclerosis

Given the immunomodulatory role of MSCs, their exosomes are being assessed extensively in experimental animal models of multiple sclerosis to impede autoimmune diseases' progression (Baharlooi et al. 2020). Employing an experimental rat model of autoimmune encephalomyelitis, Li and colleagues investigated the effect of exosomes derived from BM-MSCs on microglia polarization and inflammation in the central nervous system (Li et al. 2019). They observed that exosome treatment significantly decreased neural behavioral scores, reduced the infiltration of inflammatory cells into the central nervous system, and reduced demyelination compared to untreated experimental model rats. At molecular levels, there was a significant increase in M2-related and TGF-b IL-10, whereas M1-related TNF-a and IL-12 decreased significantly in exosome treatment. Based on these data, they proposed that therapy using exosomes derived from BM-MSCs could be an interesting alternative for treating multiple sclerosis. On the same note, experiments are underway to exploit exosomes' ability to cross the blood–brain barrier using aptamer bio-conjugated exosomes to enhance oligodendroglia cell line in vitro and reduce demyelinated lesions in the brain of experimental mice model (Hosseini et al. 2019). Intravenous administration of MSCs-exosomes also helped improve recovery in experimental mice with progressive multiple sclerosis (Laso-García et al. 2018). Despite these encouraging data, no clinical trials have been designed to assess their safety and feasibility in human patients as yet.

Conclusion

In conclusion, in this chapter, we have focused only on a few of the diseases treated with MSCs in the clinical studies after extensive characterization in experimental animal models. More recently, the same conditions are being investigated to treat

with exosomes derived from MSCs as part of the emerging cell-free therapy. Despite encouraging data from the experimental animal studies, little progress has been made using exosomes in the clinics. Most of the clinical trials with exosomes are relevant to cancer, that is, Metastatic pancreatic cancer (NCT03608631), Colon cancer (NCT01294072), Malignant ascites and pleural effusion (NCT01854866), Type 1 diabetes, that is, (NCT02138331) and acute ischemic stroke, that is, (NCT03384433).

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Mesenchymal Stromal Cells for COVID-19 Critical Care Patients

6

A Present Hope

Abdelkrim Hmadcha, Tarik Smani, Jose Miguel Sempere-Ortells, Robert Chunhua Zhao, and Bernat Soria

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A. Hmadcha (✉)
Department of Biotechnology, University of Alicante, Alicante, Spain

University of Pablo de Olavide, Sevilla, Spain

Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid, Spain
e-mail: khmadcha@upo.es

T. Smani
Cardiovascular Pathophysiology, Institute of Biomedicine of Seville (IBiS), University Hospital of Virgen del Rocío/University of Seville/CSIC, Seville, Spain

Department of Medical Physiology and Biophysics, University of Seville, Seville, Spain

Biomedical Research Networking Centers of Cardiovascular Diseases (CIBERCV), Madrid, Spain
e-mail: tasmani@us.es

J. M. Sempere-Ortells
Department of Biotechnology, Immunology Division, University of Alicante, Alicante, Spain
e-mail: josemiguel@ua.es

R. C. Zhao
Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China

Department of Cell Biology, School of Life Sciences, Shanghai University, Shanghai, China
e-mail: zhaochunhua@vip.163.com

B. Soria
University of Pablo de Olavide, Sevilla, Spain

Department of Physiology, Institute of Bioengineering-Alicante Institute for Health and Biomedical Research (ISABIAL), University Miguel Hernández School of Medicine, Alicante, Spain
e-mail: bernat.soria@umh.es

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Abstract

Despite the titanic efforts of health systems worldwide through the implementation of severe public health measures, the number of patients with the current coronavirus disease 2019 (COVID-19) has been dramatically increasing since December 2019. COVID-19 is a real threat that is currently becoming a major concern worldwide. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has a virulent infection leading to a high mortality rate. Although, the emergency use authorization of COVID vaccines has brought hope to mitigate pandemic of COVID-19, there remains a need for additional effective vaccines to deal with SARS-CoV-2, a virus characterized by its unpredictable nature, high morbidity, and rapid ability to spread and to meet the global demand and address the potential new viral variants. Still there is a significantly increased demand for the development of new therapeutic alternatives to palliate the ongoing pandemic. Actually, treating critical COVID-19 patients is challenging as no specific treatment options against SARS-CoV-2 are available. The main pathologic features of critical COVID-19 were consistent with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Therefore, regenerative, immunomodulatory, and anti-inflammatory properties of mesenchymal stromal cells (MSCs) can reduce the manifestation of cytokine storm and can restore ARDS and ALI, exhibiting an important option to be applied to critical COVID-19 patients. Here we propose MSCs as a potential alternative therapy for COVID-19 patients and discussed specific aspects of this proposed cell therapy.

Keywords

ARDS · CAR-T cells · Cell therapy · COVID-19 · Cytokine storm · Immunomodulation · Inflammation · Mesenchymal stromal cells · Organoids · SARS-CoV-2 · Stem cells

List of Abbreviations

3D	Three-dimensional
ACE2	Angiotensin-converting enzyme 2
ALI	Acute lung injuries
Ang-1	Angiopoietin-1
ARB	Angiotensin receptor blocker

ARDS	Acute respiratory distress syndrome
AT2s	Type 2 alveolar epithelial cells
CAR-T	Chimeric antigen receptor T cells
CCN1	CCN family number 1
CFTR	Cystic fibrosis transmembrane conductance regulator
CLDN1	Claudin1
COVID-19	Coronavirus disease 2019
CPE	Cytopathologic effect
Cyr61	Cysteine-rich protein 61
DEX	Dexamethasone
ECM	Extracellular matrix
EMMPRIN	Extracellular matrix metalloproteinase inhibitor (or CD147)
FDA	Food and Drug Administration
GFP	Green fluorescent protein
GI	Gastrointestinal
Gsis	γ-Secretase inhibitors
HACE2	Human angiotensin-converting enzyme 2
HCQ	Hydroxychloroquine
HE	Heme agglutinin esterase
HESCs	Human embryonic stem cells
HiPSCs	Human-induced pluripotent stem cells
HPSC	Human pluripotent stem cell
Hrsace2	Human recombinant soluble ACE2
Hs-cTnI	Highly sensitive troponin-I
HSV1	Herpes simplex virus-1
ICU	Intensive care units
IFN	Interferon
$Ifnar1^{-/-}$	C57BL/6 mice with a genetic ablation of their type I interferon receptors
IL-1α	Interleukin-alpha
IL1-β	Interleukin-beta
IL-2	Interleukin-2
$Il28r^{-/-}$	C57BL/6 mice with a genetic ablation of their type III interferon receptors
Il2rg	Interleukin-2 receptor gamma chain
Isgs	Interferon-stimulated genes
KGF	Keratinocyte growth factor
KRT18	Cytokeratin 18
LPS	Lipopolysaccharide
MAS	Macrophage activation syndrome
Mascp6	Mouse-adapted strain at passage 6
MERS	Middle East respiratory syndrome
MODS	Multiple organ dysfunction syndromes
MPA	Mycophenolic acid
MSCs	Mesenchymal stem cells
NPC	Neural progenitor cells

NSCs:	Neural stem cells
NSG mouse	NOD-SCID with null mutation in the gene encoding the il2rgl
PAMPs	Pathogen-associated molecular patterns
PDGFb	Platelet-derived growth factor subunit b
PMN	Polymorphonuclear
QNHC	Quinacrine dihydrochloride
RIG-I	Retinoic acid-inducible gene-I-like
RLU	Relative luciferase units
RM	Regenerative medicine
SARS-cov-2	Severe acute respiratory syndrome coronavirus-2
SFTPC	Surfactant protein-C
SLC10A2	Solute carrier family 10 member 2
SOS	Sinusoidal obstructive syndrome
S-protein	Spike protein
TF	Tissue factor (or CD142)
TMPRSS2	Transmembrane serine protease 2
TNFα	Tumor necrosis factor alpha
WHO	World Health Organization
WT	Wild type

Introduction

Late in December 2019, an outbreak of atypical pneumonia of unknown etiology was described in Wuhan Province in China. A novel coronavirus named "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)" was then identified as the etiologic agent (Gorbalenya et al. 2020; Wu et al. 2010). Later, the disease was designated **CO**rona **VI**rus **D**isease 2019 (COVID-19) (World Health Organization 2020). The rapid expansion of COVID-19 cases in number and geographic distribution prompted the World Health Organization (WHO) to declare a global health emergency. Containment of the disease was hindered by the lack of antiviral treatment, lack of vaccines, and asymptomatic carriers. On March 11, 2020, COVID-19 was officially classified by the WHO as a pandemic.

The WHO has declared coronavirus disease 2019 (COVID-19) a pandemic due to the rapid increase in infections worldwide. The initial outbreak occurred in Wuhan City of Hubei Province of the People's Republic of China in December 2019 and has since spread to nearly every country and territory globally. As of April 24, 2021, there have been more than 145 million confirmed cases of COVID-19 in the world, including about 3 million deaths, reported to WHO (WHO 2020). However, despite strict worldwide containment strategies and national closures in many countries, prevalence rates continue to increase with significant mortality.

Since COVID-19 affects different people in different ways, most infected people develop mild-to-moderate disease and recover without hospitalization, but a subset of patients progresses to severe illness, with a high mortality rate and limited treatment options. The clinical feature of COVID-19 varies from asymptomatic

forms to conditions involving multiorgan and systemic manifestations in terms of septic shock and multiple organ dysfunction syndromes (MODS). The primary pathologic features of critical COVID-19 were consistent with acute lung injuries (ALI) and acute respiratory distress syndrome (ARDS). The majority of infected persons is usually asymptomatic or has mild symptoms, and about 15% are affected by ARDS, of which 5% progress to multiple organ dysfunction syndromes or failure. From the point of view of prevention, evasive carriers still in the early stage of infection, and that therefore do not show any clinical manifestation of the disease, are the most infectious and the least tractable.

This pathology involves direct attacks by the virus on the cells and secondary attacks on the body after activating the immune system. This means that both the virus and the immune response can cause damage to the body, and common complications or secondary infection can occur. At the cellular level, the spike protein (S-protein) of SARS-CoV-2 interacts with cell receptors to infect target cells. SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2), triggering the endocytosis of virus particles. Consequently, ACE2 receptor would represent a potential therapeutic candidate to study SARS-CoV-2 infection mechanisms. Treating COVID-19 patients is challenging as no specific treatment options against SARS-CoV-2 are available. The current supportive but not curative treatments consist of the use of experimental medication. These include remdesivir, hydroxychloroquine, abidol, lopinavir/ritonavir, plasma from convalescent patients, antibody, and other nonspecific vaccines (Haider and Hyder 2020). Currently, remdesivir appears to be the most promising pharmacological intervention for the treatment of pneumonia caused by COVID-19.

The exact pathogenesis of the virus and the dynamics of the disease are not yet fully understood; therefore, the available treatment options are limited. These consist mainly of supportive therapies for symptomatic treatment. Several antiviral drugs (Grein et al. 2020), corticosteroids (Wang et al. 2020b; Al-Rasheed et al. 2021), convalescent plasma (Shen et al. 2020; Verma et al. 2020), and neutralizing monoclonal antibodies (Shanmugaraj et al. 2020) have been tested and have undergone different phases of clinical trials, but none have been approved explicitly for COVID-19. Another alarming fact is the report from some countries of recurrence of infection in recovered as well as vaccinated individuals, which calls into question the efficacy of available treatments (Lan et al. 2020). In the absence of a recommended treatment, observance of general principle of resorting to take preventive measures, including social distancing, hygienic precautions, and use of face masks, remains the preferred strategy (Hyder and Haider 2020).

Within this scenario, investigations have been conducted at a dizzying speed to achieve a vaccine. The pioneering manufacturer's platforms of vaccines (BioNTech-Pfizer, University of Oxford-AstraZeneca, Moderna, Johnson & Johnson, Sanofi-GlaxoSmithKline, CanSino Biologics, Inovio, Sinovac, Novavax, Gamaleya Research Institute, CureVac, Clover Biopharmaceuticals, Merck & Co.) have been able to ensure their historic and rapid development. According to WHO, as of February 18, 2021, at least seven different vaccines across three platforms have been rolled out in countries. Vulnerable populations in all countries are the highest priority for vaccination. At the same time, more than 200 additional vaccine

candidates are in development, of which more than 60 are in clinical development (García-Montero et al. 2021; Yan et al. 2021a).

The first mass vaccination program was initiated in early December 2020, and as of February 15, 2021, 175.3 million vaccine doses have been administered (Hasan et al. 2021). At least seven different vaccines (three platforms) have been developed and administered as part of the worldwide vaccination program. WHO issued an Emergency Use Listing (EUL) for the Pfizer COVID-19 vaccine (BNT162b2) on December 31, 2020. On February 15, 2021, WHO issued EUL for two versions of the AstraZeneca/Oxford COVID-19 vaccine, manufactured by the Serum Institute of India and SKBio. On March 12, 2021, WHO issued an EUL for the COVID-19 vaccine Ad26.COV2.S, developed by Janssen (Johnson & Johnson). WHO is on track to EUL other vaccine products through June.

Although many biotechnology companies have developed different vaccines and millions of people have been vaccinated to date, the complete process of safety evaluation, manufacturing, and scale-up are still under question, and longer follow-up is needed (Yan et al. 2021b; Kadkhoda 2021). As such, the development of feasible, safe, and effective therapies is extremely urgent. Therefore, increasing experimental and clinical evidence has given credibility to the claim that advanced therapies research could change the future of COVID-19 and the forthcoming emergence of virulent viruses. Notably, cell-based therapies will impact, not yet foreseen, on the present and future sequels of COVID-19. In this regard, mesenchymal stem cells (MSCs) have long been associated with the repairing and rejuvenating damaged tissues due to their broad pharmacological effects, including anti-inflammation, immunomodulation, anti-apoptosis, angiogenesis, and trans-differentiation to specific cell types. They also secrete a myriad of soluble factors and vesicles altogether involved in restoring tissue homeostasis and functionality. The efficacy of MSCs and their secretory factors has been proven in successfully reducing inflammation, dampening immune responses, and repairing lung damage in various preclinical and clinical models (Hmadcha et al. 2009). Therefore, the potential of MSC-based therapy as an option for severe or critically ill COVID-19 patients is being explored in the current scenario (Leng et al. 2020; Sánchez-Guijo et al. 2020) (Table 1).

On the one hand, recent studies focus on regenerative, immunomodulatory, and anti-inflammatory properties of mesenchymal stromal cells (MSCs) to reduce the manifestation of cytokine storm and to restore ARDS and ALI, exhibiting an important option to be applied to critical COVID-19 patients, or on MSCs secretome to treat COVID-19 pneumonia (Tang et al. 2020; Meng et al. 2020; Li et al. 2020b; Liang et al. 2020; Lanzoni et al. 2021). Other research includes the use of hematopoietic stem cells derived from umbilical cord blood, bone marrow, or mobilized peripheral blood, as well as immune chimeric antigen receptor T cell (CAR-T cell). On the other hand, the understanding of the mechanism of infection and pathogenesis are still limited. In this regard, the use of human pluripotent stem cells, both embryonic stem cells (hESCs) and induced stem cells (hiPSCs), to generate tissue-specific human organoids (lung, intestinal, liver, vascular, heart, and kidney

Table 1 Registered clinical trials using MSC-based therapy to COVID-19 (NIH-*ClinicalTrial.gov*)

NCT number	Title	Study results	Phases	URL
NCT04444271	Mesenchymal Stem Cell Infusion for COVID-19 Infection	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04444271
NCT04416139	Mesenchymal Stem Cell for Acute Respiratory Distress Syndrome Due for COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04416139
NCT04713878	Mesenchymal Stem Cells Therapy in Patients With COVID-19 Pneumonia	No results available	Not applicable	https://ClinicalTrials.gov/show/NCT04713878
NCT04429763	Safety and Efficacy of Mesenchymal Stem Cells in the Management of Severe COVID-19 Pneumonia	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04429763
NCT04898088	A Proof of Concept Study for the DNA Repair Driven by the Mesenchymal Stem Cells in Critical COVID-19 Patients	No results available	Not applicable	https://ClinicalTrials.gov/show/NCT04898088
NCT04315987	NestaCell® Mesenchymal Stem Cell to Treat Patients With Severe COVID-19 Pneumonia	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04315987
NCT04611256	Mesenchymal Stem Cells in Patients Diagnosed With COVID-19	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04611256
NCT04302519	Novel Coronavirus Induced Severe Pneumonia Treated by Dental Pulp Mesenchymal Stem Cells	No results available	Early Phase 1	https://ClinicalTrials.gov/show/NCT04302519
NCT04456361	Use of Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome Caused by COVID-19	No results available	Early Phase 1	https://ClinicalTrials.gov/show/NCT04456361
NCT04625738	Efficacy of Infusions of MSC From Wharton Jelly in the SARS-Cov-2 (COVID-19) Related Acute Respiratory Distress Syndrome	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04625738
NCT04366271	Clinical Trial of Allogeneic Mesenchymal Cells From Umbilical Cord Tissue in Patients With COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04366271

(continued)

Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04366323	Clinical Trial to Assess the Safety and Efficacy of Intravenous Administration of Allogeneic Adult Mesenchymal Stem Cells of Expanded Adipose Tissue in Patients With Severe Pneumonia Due to COVID-19	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04366323
NCT04252118	Mesenchymal Stem Cell Treatment for Pneumonia Patients Infected With COVID-19	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04252118
NCT04313322	Treatment of COVID-19 Patients Using Wharton's Jelly-Mesenchymal Stem Cells	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04313322
NCT04909892	Study of Allogeneic Adipose-Derived Mesenchymal Stem Cells to Treat Post COVID-19 "Long Haul" Pulmonary Compromise	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04909892
NCT04905836	Study of Allogeneic Adipose-Derived Mesenchymal Stem Cells for Treatment of COVID-19 Acute Respiratory Distress	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04905836
NCT04753476	Treatment of Severe COVID-19 Patients Using Secretome of Hypoxia-Mesenchymal Stem Cells in Indonesia	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04753476
NCT04336254	Safety and Efficacy Study of Allogeneic Human Dental Pulp Mesenchymal Stem Cells to Treat Severe COVID-19 Patients	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04336254
NCT04346368	Bone Marrow-Derived Mesenchymal Stem Cell Treatment for Severe Patients With Coronavirus Disease 2019 (COVID-19)	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04346368
NCT04288102	Treatment With Human Umbilical Cord-derived Mesenchymal Stem Cells for Severe Corona Virus Disease 2019 (COVID-19)	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04288102
NCT04629105	Regenerative Medicine for COVID-19 and Flu-Elicited ARDS Using Longeveron Mesenchymal Stem Cells (LMSCs) (RECOVER)	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04629105

(continued)

Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04273646	Study of Human Umbilical Cord Mesenchymal Stem Cells in the Treatment of Severe COVID-19	No results available	Not applicable	https://ClinicalTrials.gov/show/NCT04273646
NCT04371601	Safety and Effectiveness of Mesenchymal Stem Cells in the Treatment of Pneumonia of Coronavirus Disease 2019	No results available	Early Phase 1	https://ClinicalTrials.gov/show/NCT04371601
NCT04527224	Study to Evaluate the Efficacy and Safety of AstroStem-V in Treatment of COVID-19 Pneumonia	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04527224
NCT04728698	Study of Intravenous Administration of Allogeneic Adipose-Derived Mesenchymal Stem Cells for COVID-19-Induced Acute Respiratory Distress	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04728698
NCT04657458	Expanded Access Protocol on Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicle Infusion Treatment for Patients With COVID-19 Associated ARDS	No results available		https://ClinicalTrials.gov/show/NCT04657458
NCT04348435	A Randomized, Double-Blind, Placebo-Controlled Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Allogeneic Mesenchymal Stem Cell Therapy (HB-adMSCs) to Provide Protection Against COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04348435
NCT04339660	Clinical Research of Human Mesenchymal Stem Cells in the Treatment of COVID-19 Pneumonia	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04339660
NCT04428801	Autologous Adipose-derived Stem Cells (AdMSCs) for COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04428801
NCT04457609	Administration of Allogenic UC-MSCs as Adjuvant Therapy for Critically-Ill COVID-19 Patients	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04457609
NCT04382547	Treatment of Covid-19 Associated Pneumonia With Allogenic Pooled Olfactory Mucosa-derived Mesenchymal Stem Cells	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04382547

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Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04349631	A Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Autologous Mesenchymal Stem Cell Therapy (HB-adMSCs) to Provide Protection Against COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04349631
NCT04366063	Mesenchymal Stem Cell Therapy for SARS-CoV-2-related Acute Respiratory Distress Syndrome	No results available	Phase 2| Phase 3	https://ClinicalTrials.gov/show/NCT04366063
NCT04352803	Adipose Mesenchymal Cells for Abatement of SARS-CoV-2 Respiratory Compromise in COVID-19 Disease	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04352803
NCT04573270	Mesenchymal Stem Cells for the Treatment of COVID-19	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04573270
NCT04490486	Umbilical Cord Tissue (UC) Derived Mesenchymal Stem Cells (MSCs) Versus Placebo to Treat Acute Pulmonary Inflammation Due to COVID-19	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04490486
NCT04355728	Use of UC-MSCs for COVID-19 Patients	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04355728
NCT04888949	A Study of ADR-001 in Patients With Severe Pneumonia Caused by SARS-CoV-2 Infection (COVID-19)	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04888949
NCT04461925	Treatment of Coronavirus COVID-19 Pneumonia (Pathogen SARS-CoV-2) With Cryopreserved Allogeneic P_MMSCs and UC-MMSCs	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04461925
NCT04522986	An Exploratory Study of ADR-001 in Patients With Severe Pneumonia Caused by SARS-CoV-2 Infection	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04522986
NCT04903327	Study of Intravenous COVI-MSC for Treatment of COVID-19-Induced Acute Respiratory Distress	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04903327

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Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04348461	BAttLe Against COVID-19 Using MesenchYmal Stromal Cells	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04348461
NCT04565665	Cord Blood-Derived Mesenchymal Stem Cells for the Treatment of COVID-19 Related Acute Respiratory Distress Syndrome	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04565665
NCT04535856	Therapeutic Study to Evaluate the Safety and Efficacy of DW-MSC in COVID-19 Patients	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04535856
NCT04362189	Efficacy and Safety Study of Allogeneic HB-adMSCs for the Treatment of COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04362189
NCT04293692	Therapy for Pneumonia Patients Infected by 2019 Novel Coronavirus	No results available	Not applicable	https://ClinicalTrials.gov/show/NCT04293692
NCT04390152	Safety and Efficacy of Intravenous Wharton's Jelly Derived Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome Due to COVID 19	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04390152
NCT04494386	Umbilical Cord Lining Stem Cells (ULSC) in Patients With COVID-19 ARDS	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04494386
NCT04397796	Study of the Safety of Therapeutic Tx With Immunomodulatory MSC in Adults With COVID-19 Infection Requiring Mechanical Ventilation	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04397796
NCT04780685	A Phase II Study in Patients With Moderate to Severe ARDS Due to COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04780685
NCT04377334	Mesenchymal Stem Cells (MSCs) in Inflammation-Resolution Programs of Coronavirus Disease 2019 (COVID-19) Induced Acute Respiratory Distress Syndrome (ARDS)	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04377334

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Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04452097	Use of hUC-MSC Product (BX-U001) for the Treatment of COVID-19 With ARDS	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04452097
NCT04345601	Mesenchymal Stromal Cells for the Treatment of SARS-CoV-2 Induced Acute Respiratory Failure (COVID-19 Disease)	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04345601
NCT04492501	Investigational Treatments for COVID-19 in Tertiary Care Hospital of Pakistan	No results available	Not applicable	https://ClinicalTrials.gov/show/NCT04492501
NCT04390139	Efficacy and Safety Evaluation of Mesenchymal Stem Cells for the Treatment of Patients With Respiratory Distress Due to COVID-19	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04390139
NCT04798716	The Use of Exosomes for the Treatment of Acute Respiratory Distress Syndrome or Novel Coronavirus Pneumonia Caused by COVID-19	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04798716
NCT04276987	A Pilot Clinical Study on Inhalation of Mesenchymal Stem Cells Exosomes Treating Severe Novel Coronavirus Pneumonia	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04276987
NCT04392778	Clinical Use of Stem Cells for the Treatment of Covid-19	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04392778
NCT04467047	Safety and Feasibility of Allogenic MSC in the Treatment of COVID-19	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04467047
NCT04798066	Intermediate Size Expanded Access Protocol Evaluating HB-adMSC's for the Treatment of Post-COVID-19 Syndrome	No results available		https://ClinicalTrials.gov/show/NCT04798066
NCT04909879	Study of Allogeneic Adipose-Derived Mesenchymal Stem Cells for Non-COVID-19 Acute Respiratory Distress Syndrome	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04909879
NCT04398303	ACT-20 in Patients With Severe COVID-19 Pneumonia	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04398303

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Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04361942	Treatment of Severe COVID-19 Pneumonia With Allogeneic Mesenchymal Stromal Cells (COVID_MSV)	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04361942
NCT03042143	Repair of Acute Respiratory Distress Syndrome by Stromal Cell Administration (REALIST) (COVID-19)	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT03042143
NCT04537351	The MEseNchymal coviD-19 Trial: MSCs in Adults With Respiratory Failure Due to COVID-19 or Another Underlying Cause	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04537351
NCT04437823	Efficacy of Intravenous Infusions of Stem Cells in the Treatment of COVID-19 Patients	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04437823
NCT04269525	Umbilical Cord (UC)-Derived Mesenchymal Stem Cells (MSCs) Treatment for the 2019-novel Coronavirus (nCOV) Pneumonia	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04269525
NCT04602442	Safety and Efficiency of Method of Exosome Inhalation in COVID-19 Associated Pneumonia	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04602442
NCT04447833	Mesenchymal Stromal Cell Therapy For The Treatment Of Acute Respiratory Distress Syndrome	No results available	Phase 1	https://ClinicalTrials.gov/show/NCT04447833
NCT04371393	MSCs in COVID-19 ARDS	No results available	Phase 3	https://ClinicalTrials.gov/show/NCT04371393
NCT04491240	Evaluation of Safety and Efficiency of Method of Exosome Inhalation in SARS-CoV-2 Associated Pneumonia.	Has results	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04491240
NCT04333368	Cell Therapy Using Umbilical Cord-derived Mesenchymal Stromal Cells in SARS-CoV-2-related ARDS	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04333368
NCT04299152	Stem Cell Educator Therapy Treat the Viral Inflammation in COVID-19	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04299152

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Table 1 (continued)

NCT number	Title	Study results	Phases	URL
NCT04524962	Study of Descartes-30 in Acute Respiratory Distress Syndrome	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04524962
NCT04541680	Nintedanib for the Treatment of SARS-Cov-2 Induced Pulmonary Fibrosis	No results available	Phase 3	https://ClinicalTrials.gov/show/NCT04541680
NCT04466098	Multiple Dosing of Mesenchymal Stromal Cells in Patients With ARDS (COVID-19)	No results available	Phase 2	https://ClinicalTrials.gov/show/NCT04466098
NCT04445220	A Study of Cell Therapy in COVID-19 Subjects With Acute Kidney Injury Who Are Receiving Renal Replacement Therapy	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04445220
NCT04341610	ASC Therapy for Patients With Severe Respiratory COVID-19	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04341610
NCT04400032	Cellular Immuno-Therapy for COVID-19 Acute Respiratory Distress Syndrome	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04400032
NCT04684602	Mesenchymal Stem Cells for the Treatment of Various Chronic and Acute Conditions	No results available	Phase 1| Phase 2	https://ClinicalTrials.gov/show/NCT04684602

organoids) may provide a next-generation cellular model for investigating viral infection and drug screening. Altogether, the ultimate goal of all these strategies is to achieve a definitive and efficient therapy for COVID-19.

Mechanism of Infection and Immune Response

Several types of coronavirus are known to have the potential for human infection; only six are known to cause disease in humans: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV (Skariyachan et al. 2019; Bonilla-Aldana et al. 2020). The 2019-nCoV, a single-stranded RNA virus, is closely related to SARS-CoV that emerged in 2003–2004 and caused an epidemic disease (Racaniello 2016). The virus was provisionally designated 2019-nCoV and later given the official name SARS-CoV-2 (Gorbalenya et al. 2020). SARS-CoV-2 was characterized as a beta-coronavirus

and recognized as the seventh discrete coronavirus species capable of causing human disease (Zhu et al. 2020a). The virion nucleocapsid consists of an RNA genome complexed with a nucleoprotein and is enveloped by a phospholipid bilayer. This bilayer is covered by two types of spike proteins: protein S, which is present in all known coronaviruses and forms peplomers on the surface, which gives it a corona solar appearance, and protein hemagglutinin esterase (HE), which is present in only a few types of coronaviruses. The spike S protein interacts with the host angiotensin-converting enzyme 2 (ACE2) receptor protein, resulting in membrane fusion with subsequent release of the viral genome into the cell, or clathrin-dependent and clathrin-independent endocytosis of the virus (Li et al. 2020a).

Moreover, the ACE2 receptor is widely expressed by human cells, particularly in the lungs by type 2 alveolar epithelial cells (AT2s) and capillary epithelial cells, and cells of the heart, kidney, and intestine. In addition, two proteases, transmembrane serine protease 2 (TMPRSS2) and extracellular matrix metalloproteinase inhibitor (EMMPRIN or CD147), have been reported to be essential for virus entry into the host cell (Chen et al. 2020). The damage to the lungs is caused by the virus either directly, by the destruction of AT2s and capillary endothelial cells, which disrupts the renin-angiotensin system, or indirectly, by dampening the immune response (Jin et al. 2020). The precise pathogenesis of this particular virus remains unknown. Most of the information on the cycle of infection and subsequent immune response is primarily derived from the SARS and MERS coronaviruses due to the correlation in the clinical features of patients with COVID-19 with these viral infections (Guan et al. 2020; Huang et al. 2020).

Upon infection, the virus is recognized by the innate immune system through pathogen-associated molecular patterns (PAMPs), which in this case is the genomic RNA of the virion. This leads to activation of the NFkB pathway and the IRF3 pathway, which results in the expression of type I interferon (IFN). IFN then activates the JAK/STAT pathway and induces the expression of IFN-stimulated genes (ISGs) that have antiviral activity (Prompetchara et al. 2020).

Successful viral clearance and amelioration of the clinical manifestations of the disease depend on this effective immune response. However, the virus can evade IFN- and ISG-mediated killing and often results in a delayed IFN response. This results in the infiltration of hyper-inflammatory neutrophils and macrophages into the lung site, along with pro-inflammatory cytokines, mainly IL-1b, IL-2, IL-6, IL-7, IL-8, IL-17, G-CSF, GM-CSF, CCL3, MCP1, and TNF (Cao 2020). This so-called cytokine storm is a result of the innate response (neutrophils and macrophages). Moreover, hyperactivation of T lymphocytes (especially the Th1 response) is actually responsible for pulmonary dysfunction and abnormalities such as pneumonitis, ARDS, respiratory failure, viral sepsis, and organ failure. Elevated pro-inflammatory cytokines also induce the synthesis of hyaluronan synthase 2, which produces hyaluronan in the lungs, leading to the characteristic opacity or fluid accumulation in the lungs (Shi et al. 2020).

In critical cases, the virus can also enter the peripheral blood (viremia) and translocate to other target organs expressing the ACE2 receptor, such as the heart, kidney, and intestines, resulting in multiple organ dysfunctions. Thus, there is a great

need to discover the specific virulence mechanisms during which cell and tissue injury occurs. As it is not always possible to capture the underlying mechanisms of pathophysiology in humans, several modeling methods have been developed, including 3D-engineered organoids derived from pluripotent stem cells (ESCs and iPSCs), different types of stem cells, and animals (Sun et al. 2020; Yang et al. 2020; Youk et al. 2020; Zheng et al. 2021; Boudewijns et al. 2020).

The Rationale for the Clinical Use of MSCs for COVID-19 Patients

MSCs administration tends to unbalance the pro-inflammatory cytokines, immune cells, and tissue damage toward an anti-inflammatory and regenerative microenvironment. MSCs have been widely used in cell-based therapy, from basic research to clinical trials (Acosta et al. 2013; Capilla-González et al. 2018; Soria-Juan et al. 2019). Safety and efficacy have been avidly documented in many clinical trials, especially in both systemic and local immune-mediated inflammatory diseases, such as GvHD, Crohn's complex fistula, and type 2 diabetes complications (Soria-Juan et al. 2019; García-Gómez et al. 2010; Herreros et al. 2012). MSCs play a positive role mainly in two ways: immunomodulatory effects and anti-inflammatory abilities both linked to regeneration (Pacienza et al. 2017; Lee and Kang 2020). MSCs, when activated, can secrete many types of cytokines by paracrine secretion or make direct interactions with immune cells (Leng et al. 2020).

MSCs may act as suppressors of the cytokine storm, specifically through IL-1 blockade. Recent data support that IL-1 receptor antagonist, a naturally occurring antagonist of IL-1α/IL1-β signaling pathways, has been attributed to the immunosuppressive effects of MSCs (Harrell et al. 2020). So, IL-1 blockade seems to activate MSCs toward anti-inflammatory phenotype able of releasing anti-inflammatory cytokines, of increasing Treg, and of favoring polarization of M1 (pro-inflammatory) macrophages into M2 (anti-inflammatory), which could contribute to revascularization and regeneration of lung tissue (Varghese et al. 2017).

In summary, MSCs tend to unbalance the pro-inflammatory cytokines, immune cells, and tissue damage toward an anti-inflammatory and regenerative microenvironment (Fig. 1).

This is why these cells have been proposed for use in pulmonary sepsis and cystic fibrosis. They are safe when used for ARDS (Wilson et al. 2015). The intravenous route is the most appropriate for the current intensive care unit (ICU) setting. Additionally, MSCs of any origin injected intravenously are rapidly located in the pulmonary microcirculation network because the cells are significant than the diameter of the capillaries, based on previous data in preclinical animal models. They can also be captured by local macrophages, which subsequently stimulate MSCs to produce IL-10, indirectly providing a source of immunosuppressive cytokines (Argibay et al. 2017). Altogether, MSCs have been successfully tested in other inflammatory diseases. Preclinical data suggest that they may be beneficial in patients with COVID-19 with severe pulmonary inflammation and oxygen therapy (mechanical ventilation) without excluding their use in earlier stages of the disease.

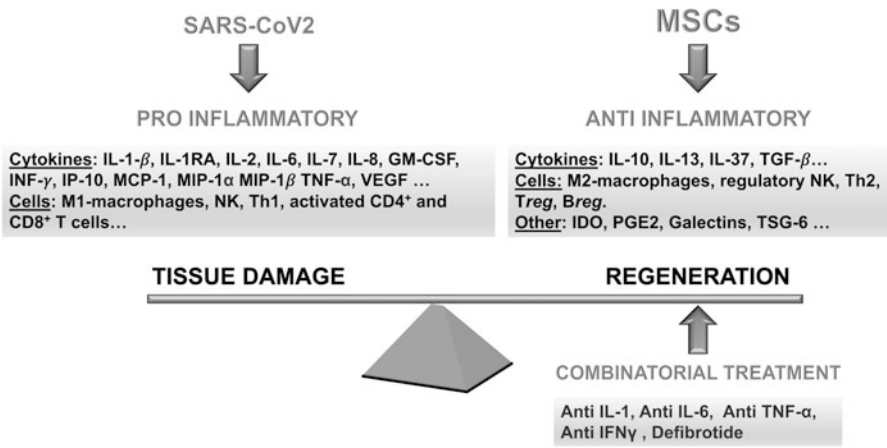

Fig. 1 Schematic of the anti-inflammatory effects of mesenchymal stem cells-derived exosomes, secretome, and combinatory treatment on lung inflammation and tissue damage caused by COVID-19. **Abbreviations:** Breg, regulatory B cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IDO, indoleamine-pyrrole 2,3-dioxygenase; IFNγ, interferon gamma; IL-1RA, interleukin-1 receptor antagonist; IL, interleukin; IP-10, interferon gamma-induced protein 10 (or CXCL10, C-X-C motif chemokine ligand 10); M1-macrophages, classically activated macrophages; M2-macrophages, alternatively activated macrophages; MCP, monocyte chemoattractant protein; MIP-1, macrophage inflammatory protein 1; NK, natural killer; PGE2, prostaglandin E2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Treg, regulatory T cell; TGF-β, transforming growth factor-beta; Th1, T helper type 1; Th2, T helper type 2; TNFα, tumor necrosis factor-alpha; TSG-6, stimulated gene-6; VEGF, vascular endothelial growth factor

Progress in MSC-Based Therapy for COVID-19

MSCs have been widely used for their capacity to differentiate into diverse cell lineages, migration, and secretion of cellular regulators (secretome), together with their immunosuppressive and immunomodulatory potential. Moreover, their isolation is almost easy and presents no major ethical problems, making them the most suitable stem cells among many others (Larijani et al. 2015).

On the one hand, adipose tissue, umbilical cord, bone marrow, and blood are some important sources of MSCs. Although adipose tissue-derived MSCs have been shown to have more exciting results initially, the best source of stem cells still needs to be found (Gentile and Sterodimas 2020; Song et al. 2021). On the other hand, through their impacts on T and B cells, macrophages, and dendritic cells, they help establish a tolerogenic environment leading to an optimal therapeutic condition (Wang et al. 2018; Lee and Song 2018). Consequently, by inhibiting T- and B-cell proliferation and successfully regulating pro-inflammatory cytokines to optimize the microenvironment for intrinsic recovery, MSCs can reduce the cytokine storm. Moreover, MSCs can restrict the infiltration of innate immune system cells, consequently decreasing the secretion of inflammatory cytokines, which may indirectly

attenuate the cytokine storm (Zhu et al. 2020b; Ellison-Hughes et al. 2020; Song et al. 2021; Jeyaraman et al. 2021).

Under COVID conditions, a few days after infusion of MSCs, immune cells related to the cytokine storm are shown to decrease. Increased levels of lymphocytes and regulatory dendritic cells along with decreased levels of CRP, IL-1, IL-6, IL-12, IFN-γ, and TNF are also other results of this type of MSC-based therapy. MSCs can also deliver antimicrobial peptides and anti-inflammatory cytokines (Rajarshi et al. 2020; Wang et al. 2020a; Leng et al. 2020). In addition to these anti-inflammatory characteristics, the secretion of IL-10 and some growth factors, together with their regenerative and repair capacity, renders MSCs a potent therapeutic tool for lung repair and treatment of ARDS (Azmi et al. 2020). Administration of MSCs has also been shown to have beneficial effects in conditions like sepsis and septic shock due to their ability to normalize inflammatory biomarkers, oxygen saturation, and pulmonary improvements. For sepsis condition, umbilical cord Wharton's jelly-derived MSCs are mentioned as the best source of MSCs due to their effectiveness and acceptability (Laroye et al. 2020).

Furthermore, the gene expression profile showed that MSCs were negatively expressed angiotensin-converting enzyme 2 (ACE2-), an essential protein for viral infection along with transmembrane serine protease 2 (TMPRSS2-), which indicated that MSCs are free from COVID-19 infection (Leng et al. 2020; Hernandez et al. 2021). In this regard, the FDA has confirmed the safety and efficacy of MSCs for widespread application for COVID-19 cases (Choudhery and Harris 2020; Kavianpour et al. 2020). Despite the aforementioned benefits on the administration of MSCs, there are still some challenges; MSCs-related characteristics regarding their dosage, route of administration, frequency, and location of MSCs in the damaged sites have posed some limitations. Ethical concerns that remain, along with the lack of standardized protocols in the preparation and isolation processes, are other challenges.

Another primary concern about MSCs therapy is their side effect of increased hypercoagulability (Jeyaraman et al. 2021). Therefore, in accordance with the increased risk of thrombosis, cell-free therapies, including MSCs secretome and MSCs extracellular vesicles, appear to be an interesting treatment approach for COVID-19 having no risk of mutagenesis and/or tumorigenicity. Exosomes harbor different types of microRNAs and mRNAs and various protein components and have a lower risk of escort and lower transmission of infection. However, a clearer understanding of the dose, timing, and route of administration of the cells is needed. Their ability for nebulized administration (Hmadcha et al. 2020; Aguilera et al. 2021) and more extended storage periods also make them promising alternative therapeutic approaches (Maron-Gutierrez and Rocco 2020; Kheirkhah et al. 2021).

One of the first clinical trials to study the efficacy of intravenous infusion of MSCs in ten patients with confirmed moderate, severe, or critical forms of COVID-19 aged 45–75 years was conducted in China (Chinese Clinical Trial Registry – ChiCTR2000029990). Seven patients (one with the critical, four with the severe, and two with the moderate form of the disease) received an intravenous infusion of MSCs; three patients with the severe disease received a placebo. Two days after

MSCs infusion, all seven patients showed a significant improvement in lung function and a decrease in the expression of disease symptoms. All seven patients who received MSCs recovered and were discharged 10 days after the procedure. Due to the immunosuppressive properties of MSCs, after infusion, they contributed to a significant decrease in the level of pro-inflammatory cytokines and chemokines in serum, which attracted a lower number of mononuclear cells/macrophages to the damaged lungs. At the same time, MSCs promoted the recruitment of many regulatory dendritic cells (DCs) to the site of inflammation. In addition, there was an increase in IL-10 and vascular endothelial growth factor (VEGF) levels in this trial, which may contribute to lung recovery. Among the three patients in the control group (who received a placebo), one died, the second developed ARDS, and the third remained in severe condition (Leng et al. 2020). Since then and over a year, many clinical trials have been launched with different types of MSCs to treat COVID-19 disease.

Other Cell-Based Innovation for COVID-19

Cell therapy is emerging as one of the most promising strategies for regenerating damaged or failed tissues and organs in the healthcare system. In this regard, in addition to the extensive use of MSCs of both autologous and allogeneic origin, there is a wide range of treatments using various cell types (e.g., T cells, NK lymphocytes, and different stem cells). In this context, the adoptive T-cell therapy approach or CAR-T cell therapy as a type of immunotherapy has proven effective against some infections and diseases. In this case, T cells are extracted from the patient's immune system (autologous source) and sent to the laboratory for genetic modification. The modified cells are then reinfused into the patient (Bonifant et al. 2016; Maus and Levine 2016; Seif et al. 2019). Despite the impressive efficacy of CAR-T cell therapy in treatment, it has several serious side effects, including cytokine release syndrome (CRS) and neurological difficulties. Immediate-onset CRS tends to be a cytokine storm (Chen et al. 2019; Hong et al. 2021). Currently, T-cell therapy has also shown promise in immunosuppressed individuals as a preventive measure against COVID-19. Thus, peripheral blood cells from convalescent subjects who had been at risk for the virus were used (Keller et al. 2020).

Regulatory T-cell-related strategies have also been suggested as treatment approaches for disease management based on their ability to inactivate innate/adaptive immunity through inhibitory molecules (Stephen-Victor et al. 2020). In addition, antigen-specific modified/unmodified T-cell transfer has shown promising results in treating different disorders by reconstituting T-cell subsets (effector/memory cells). In this context, it is mentioned that adoptive T-cell therapy by transferring immune subsets of T cells has therapeutic benefits that may be the same as the characteristics of adult tissue stem cells. However, the required high maintenance of memory T cells and engraftment processes may create some limitations (Busch et al. 2016). In this regard, specific COVID-19-related T cells (within CD45RA memory T cells) have been recognized that can be feasibly received by depleting CD45RA

from convalescent donors. These cells can provide a cell population for the condition of lymphopenia along with rapid reactions to infection. Memory T cells can respond quickly to infection and provide long-term immune protection to reduce the severity of COVID-19 symptoms. Also, CD45RA memory T cells confer protection from other pathogens encountered by the donors throughout their life (Ferreras et al. 2021). CD45RA memory T cells also provide immunity against probable secondary infections that may be found in COVID-19 hospitalized individuals (Ferreras et al. 2021). HLA-matched cytotoxic T cells isolated from convalescent patients are other promising approaches for treating COVID-19, as are EBV-specific cytotoxic T cells used for EBV^{+}-related lymphomas (Hanley et al. 2020).

Another promising candidate for a significant advance has been natural killer (NK) cell therapy. In this case, autologous or allogeneic origins can be used to create pure populations of NK cells. The use of allogeneic NK cells as a platform for CAR engineering has been augmented by the limitations of autologous NK cells (such as a diminished effector role and the requirement for a patient-specific stock) (Veluchamy et al. 2017; Daher and Rezvani 2018). Given that the decrease in NK cell numbers may be related to the severity of COVID-19 infection, some clinical trials used engineered NK cells to help combat COVID-19 (Market et al. 2020; van Eeden et al. 2020). However, the use of NK cells also has many drawbacks that may clinically hinder their efficacy. Their short lifespan (due to the lack of cytokine support), low cell number, and vulnerability to immunosuppressed status could limit their trafficking and function (Nayyar et al. 2019; Liu et al. 2021).

Side Effects of Mesenchymal Stem Cell-Based Therapy

Although the safety and efficacy of MSCs infusion have been demonstrated in hundreds of clinical trials, this treatment can lead to potential complications (Acosta et al. 2013; Capilla-González et al. 2018; Soria-Juan et al. 2019), and possibly it is the time for combinatory therapies. Preclinical studies have shown that the lungs act as a filter that retains most of the cells injected intravenously (Zhang et al. 2020). Numerous critically ill COVID-19 patients are in a hypercoagulable procoagulant state. Hence, they are at high risk for disseminated intravascular coagulation, thromboembolism, and thrombotic multiorgan failure, another cause of high lethality of the infection. It remains unclear whether intravenous (IV) infusion is a safe and effective route of MSCs infusion for COVID-19 patients. This information is important as MSC-based products express variable levels of highly procoagulant tissue factor (TF or CD142), which compromises the hemocompatibility and safety profile of the cells. Of potential concern is that intravenous infusions of poorly characterized MSC products with uncontrolled (high) TF (CD142) expression may trigger blood clotting in COVID-19 subjects and other vulnerable patient populations and further promote the risk of thromboembolism.

By contrast, well-characterized products with robust manufacturing procedures and optimized clinical delivery modes hold great promise for improving COVID-19 patients by exerting their beneficial immunomodulatory effects, inducing tissue

repair and organ protection. While the need for MSCs therapy for COVID-19 subjects is evident, integrating innate and adaptive immune compatibility testing into current cell, tissue, and organ transplantation guidelines is critical for safe and effective therapies. Thus, it is essential to use only well-characterized and safe MSCs for even the most urgent and experimental treatments (Moll et al. 2020). Because the COVID-19 patients suffer a prothrombotic state, concomitant use of heparin and defibrotide, a drug used in sinusoidal obstructive syndrome (SOS) after hematopoietic stem cell transplantation, has been proposed. Defibrotide is a mixture of single-stranded oligonucleotide aptamers with multi-target pharmacology limiting endothelial cell activation (Pescador et al. 2013). Given its antithrombotic, anti-TNFα, anti-atherosclerotic, etc., the US Food and Drug Administration (FDA) approved its use in SOS. It will be consistent both to block the cytokine storm and prevent pulmonary thromboembolism. With HIV infections, we learned that combinatorial therapies show higher effectiveness in controlling the disease. Case reports, pilot studies, and well-designed clinical trials are needed to fight this pandemic. Now and when it comes back.

Unmet Challenges of Adoptive MSCs Therapy

Despite the promising preliminary results, specific challenges demand attention before MSCs can be adopted at a larger scale in treating coronaviruses-induced infections. These challenges include the study design, source of MSCs, route of administration, dosage requirements, and their laboratory preparation and manipulation (Escacena et al. 2015; Soria-Juan et al. 2019; García-Bernal et al. 2021).

Study Design

We believe that most of the trials that have been registered utilizing MSCs or their derived products do not have any appropriate control to conclusively determine the efficacy of cellular or cell-free therapy (Table 1). In most of these cases, MSCs are used in combination with adjunct antiviral drugs and supportive therapy. In such cases, it would become almost impossible to determine if the observed clinical improvements are actually attributed to MSCs or not. Therefore, a strategy including an appropriate control should be included in the design of such trials along with a greater sample size to validate the clinical efficacy of stem cells technology fully.

Source of Cells

Different tissue sources, whether adult- or fetus-associated tissues, like adipose tissue, umbilical cord, dental pulp, and bone marrow, vary in their capacity to generate MSCs. Therefore, choosing an ideal source for harvesting MSCs and subsequently generating cell-free products is equally important as cell-free therapy

is fast emerging as a therapeutic option (Haider and Aslam 2018). MSCs tissue sources, like umbilical cord and adipose tissue, are easily accessible without discomfort to the donor and generate a greater amount of MSCs with equivalent differentiation potential from the same amount of tissue in comparison to bone marrow, which incidentally is more invasive.

Route of Cell Administration

Another major factor to consider here is their route of administration. MSCs and their products can either be systemically or locally injected. There are only limited studies that have compared the different routes of MSCs administration and have reported different outcomes. Therefore, it is difficult to conclude which route can be considered as the safest and most effective (Antunes et al. 2014; Cardenes et al. 2019). For COVID-19 disease, MSCs can be administered both systemically and locally with equal efficacy as both of these routes would result in their delivery first to the lungs only. However, in the case of cell-free products, like exosomes, delivery directly to the lungs via intranasal or intratracheal route is a more tenable option as systemic delivery often leads to a substantial loss in the amount of these products, mainly due to the activity of circulatory proteases and their distribution to the liver and spleen first, thus calling for booster doses (Gardin et al. 2020; Mahajan and Bhattacharyya 2021).

Dosage Strategies

The number of MSCs required per dose and the total number of doses required are quite crucial in determining the treatment outcome. Based on the previous studies, it is estimated that approximately 4×10^8 MSCs are required for every patient regardless of the clinical indication (Olsen et al. 2018). The trials registered for COVID-19 have reported varied dosages, with an average of 1×10^6 cells/kg body weight up to 2–5 times to this average dose, but the actual number of MSCs required to produce such doses is not mentioned in any report, which needs to be optimized. Furthermore, while MSCs can be injected directly, the products like exosomes need to be prepared into stable formulations for their delivery to the patients (Gardin et al. 2020; Mahajan and Bhattacharyya 2021).

Risks Associated with Stem Cell Therapy

While MSCs and their products have proven beneficial in the current scenario, we should not overlook the risks associated with stem cell therapy. Many stem cells clinics have opened up in recent years, marketing unethical and unauthorized stem cell treatment for various ailments, including COVID-19, by feeding on people's fears and anxieties (Turner 2020). These less than clinical-grade stem cells and unlicensed stem cell treatments are potentially dangerous to the general public and undermine the efforts at determining stem cells efficacy in clinical trials. Undoubtedly, the use of

MSCs and their factors has proven to be quite promising in the current scenario but is mainly dependent on the functional quality and integrity of stem cells. Therefore, stringent regulatory control should be maintained for the manufacturing and distribution of these products (see ▶ Chap. 3, "Considerations for Clinical Use of Mesenchymal Stromal Cells" of this book for review on clinical use of MSCs).

Concluding Remarks

Considering the prevalence of COVID-19 and its complications, such as cytokine storm, which is followed by ARDS and death of patients, finding a way to treat and improve patients is of great importance. As previously mentioned, currently available vaccines are not a cure for COVID-19; there is no specific therapy for this virus, and supportive therapies as well as nonspecific antiviral drugs are mainly used for this purpose. Cell-based therapy is a modern method to treat various diseases. Recently, a number of studies have been conducted to treat COVID-19 using stem cells, suggesting the application of MSCs or immune cells such as T, CAR-T, or NK cells. Accordingly, the safety and immunomodulatory role of MSCs in ARDS has been approved. The MSCs can secrete factors that improve the pulmonary microenvironment, inhibit immune system over-activation, promote tissue repair, rejuvenate alveolar epithelial cells, inhibit counteract pulmonary fibrosis, or improve function in lung tissue damaged by SARS-CoV-2 infection.

Nonetheless, many issues related to the application of MSCs, such as the ideal dose and optimal timing of administration, need further study. Of note, in several animal models of human disease, the use of MSC-secreting exosomes has been claimed to mimic the beneficial effects of MSCs in antiviral therapy against the influenza virus by reducing virus replication in the lungs and virus-induced release of pro-inflammatory cytokines, which highlights the great potential of cell-free-based therapies. In addition, considering the impossibility of studying the detailed mechanism of pathogenicity and the sequence of suggested drugs or vaccine candidates in human beings, these significant steps toward cell-based therapies in the SARS-CoV-2 field of study should be continued. Ongoing experimental studies and randomized trials will play an essential role in elucidating the therapeutic potential of MSCs, leading to a better understanding of how MSCs interact with SARS-CoV-2-infected lung tissue. Although current progress on COVID-19 vaccinations is promising, the world population will have to continue to adapt to the "new normal" and practice social distancing and hygienic measures, at least until an effective cure is available to the general public.

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Mesenchymal Stem Cell Therapy for Inflammatory Bowel Disease

7

Mikhail Konoplyannikov, Oleg Knyazev, Peter Timashev, and Vladimir Baklaushev

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M. Konoplyannikov (✉)
Federal Research Clinical Center of Specialized Medical Care and Medical Technologies of the FMBA of Russia, Moscow, Russia

Department of Advanced Biomaterials, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia
e-mail: konoplyannikov_m_a@staff.sechenov.ru

O. Knyazev
Department of Inflammatory Bowel Diseases, Moscow Clinical Scientific Center Named After A. S. Loginov, Moscow, Russia

Department of Inflammatory Bowel Diseases, State Scientific Centre of Coloproctology Named After A. N. Ryzhih, Moscow, Russia

P. Timashev
Department of Advanced Biomaterials, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia

Laboratory of Clinical Smart Nanotechnologies, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia

Department of Polymers and Composites, N. N. Semenov Institute of Chemical Physics, Moscow, Russia

Chemistry Department, Lomonosov Moscow State University, Moscow, Russia

V. Baklaushev
Federal Research Clinical Center of Specialized Medical Care and Medical Technologies of the FMBA of Russia, Moscow, Russia

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Abstract

Inflammatory bowel disease (IBD) belongs to the group of diseases characterized by idiopathic inflammation of the gastrointestinal organs. Two basic IBD types are distinguished: ulcerative colitis and Crohn's disease. The IBD symptoms including vomiting and diarrhea, abdominal pain, rectal hemorrhage, and anemia have a significant negative impact on the general patient's state of health. More than four million people in the USA and Europe suffer from IBD, while the general incidence of this disease in the developed countries exceeds 0.5% of the population. Besides, IBD is associated with a significant risk of colitis-associated malignancy. In the last decades, considerable progress has been achieved in the IBD therapy due to application of drugs suppressing the local gastrointestinal tract inflammation, such as antibodies to TNF-α (infliximab and adalimumab), corticosteroids, salicylates, etc. At the same time, this strategy, unfortunately, does not result in the repair of the damaged tissues, primarily ulcers of the colon, in many IBD patients. To achieve the mucosa healing and stable remission in IBD patients, novel approaches are required, cell therapy, actively used since the beginning of 2000s, being one of them. In our book chapter, we discuss the advantages and problems of application of mesenchymal stem cells (MSCs) which are most actively used in the cell therapy of IBD. The results of the most important preclinical and clinical studies are covered.

Keywords

Clinical trials · Inflammatory bowel disease · Ulcerative colitis · Crohn's disease · Cell therapy · Mesenchymal stem cells · Regenerative medicine

Abbreviations

5-ASA	5-Aminosalicylic acid
APC	Antigen-presenting cells
ASC	Adipose tissue–derived stem cells
AZA	Azathioprine
BM	Bone marrow
CCL-2	C-C-chemokine ligand 2
CD	Crohn's disease
CDAI	Crohn' Disease Activity Index
CI	Confidence interval
DC	Dendritic cells
DSS	Dextran sodium sulfate
GCS	Glucocorticosteroids

GI	Gastrointestinal
HBI	Harvey-Bradshaw Index
HGF	Hepatocyte growth factor
HLA-G	Human leukocyte antigen-G
hUC	Human umbilical cord
I/A	Intra-arterial
I/V	Intravenous
IBD	Inflammatory bowel disease
IBDQ	Inflammatory Bowel Disease Questionnaire
ICG	Lindocyanine green
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
miR	MicroRNA
MMP	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
NK-cells	Natural killer cells
PDAI	Perianal Disease Activity Index
PGE2	Prostaglandin E2
PSC	Primary sclerosing cholangitis
RR	Relative risk
SMA	Smooth muscle actin
SVF	Stromal vascular fraction
TGF-β	β-transforming growth factor
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
UC	Ulcerative colitis

Introduction

Inflammatory bowel disease (IBD) is a group of chronic inflammatory conditions of the gastrointestinal (GI) tract characterized by the augmented immune response of the mucosa. Crohn's disease (CD) and ulcerative colitis (UC) are the two basic types of IBD. Long-lasting IBD results in GI tract damage. CD may affect any part of the GI tract from the mouth to the anus. The terminal part of the small intestine (ileum) is most frequently affected near the place where it joins the large intestine. CD may manifest itself in the form of "patches," involving some parts of the GI tract and leaving the other parts intact. The inflammation in CD may spread through the whole colon wall thickness (Sairenji et al. 2017). In UC, only the colon and rectum are affected. The inflammation appears only in the innermost layer of the colon mucosa. It usually starts in the rectum and lower parts of the colon, but may also spread

continuously and affect the entire large intestine. IBD shares some symptoms such as persistent diarrhea, abdominal pain, rectal bleeding/bloody stool, weight loss, and fatigue. In some cases, it is difficult to determine whether a patient has CD or UC. Such cases are classified as indeterminate colitis (Guindi and Riddell 2004).

The exact cause of IBD is unknown, but there is an assumption that it results from a defective immune system. The immune system of an IBD patient wrongly reacts to the environmental triggers that cause the GI tract inflammation. Such a wrong reaction of the immune system arises, supposedly, in people with a corresponding family history who inherited genes determining the susceptibility to IBD (Khor et al. 2011).

More than four million people in the USA and Europe suffer from IBD, while the general incidence of this disease in the developed countries exceeds 0.5% of the population. Seventy thousand new IBD cases are diagnosed yearly in the USA only, and in general, the yearly financial burden of IBD in the USA exceeds 31 billion dollars (CCFA 2014; GBD 2020). The majority of patients receive the diagnosis of IBD at the age of less than 35 years. In particular, 80,000 children suffer from IBD in the USA. These lifelong chronic conditions essentially affect the quality of life and medical expenses of patients. Besides, IBD patients are susceptible to the risk of developing of other serious diseases such as colon cancer, thrombosis, and primary sclerosing cholangitis (PSC).

In some cases, surgical removal of the damaged GI parts is required for the therapy of severe IBD forms. However, due to the achieved success of the drug therapy of IBD, it has been generally used in the last decades, with five basic types of drugs (CCFA 2014).

Aminosalicylates such as sulfasalazine, balsalazide, mesalamine, and olsalazine administered per os or rectally reduce the colon wall inflammation and are applied primarily for the UC treatment. At the same time, they are less efficient in the CD treatment.

Corticosteroids, such as prednisone, prednisolone, and budesonide, keep the immune system under control. Therefore, they are efficient in the short-term management of exacerbations. But unfortunately, their side effects include infections, weight gain, sleep disorders, etc.

Immunomodulators, such as azathioprine, 6-mercaptopurine, and methotrexate, influence the immune system activity; they are toxic and usually used to sustain the remission in those patients who do not respond to other drugs, or respond to steroids only.

Antibiotics, such as ciprofloxacin and metronidazole, are of moderate use in treating CD patients with the affected colon or perianal region. In particular, antibiotics are administered in the case of infections, e.g., abscesses.

TNF inhibitors include adalimumab, certolizumab pegol, golimumab, and infliximab. These drugs have a pronounced anti-inflammatory effect and are used in the therapy of patients suffering from severe forms of IBD in the absence of a satisfactory and sound effect from the standard treatment. However, the application of these drugs, regretfully, is not always efficient, as well. In particular, the long-term infliximab administration has shown that up to one-third of patients do not respond

to the anti-cytokine therapy, either due to primary resistance to the drug or the development of secondary resistance (Magro and Portela 2010). Besides, severe complications may occasionally emerge, including bacterial, viral, and fungal infections, increased risk of lymphoma, colorectal cancer, and other oncological diseases.

IBD Therapy with MSCs

According to estimates, application of contemporary methods of IBD therapy leads to a 20–30% rate of remission, with a maximum of 50% when using a combinatorial therapy approach (Ocansey et al. 2020). Furthermore, cell therapy has shown to be very effective and extremely promising in treating IBD (Cassinotti et al. 2012; Irhimeh and Cooney 2016; Lopez-Santalla et al. 2020). Therefore, the use of mesenchymal stem cells (MSCs) is of particular interest regarding this approach.

MSCs Properties

MSCs are multipotent stromal cells which may be derived from the bone marrow, adipose tissue, dental pulp, skeletal muscle, etc. (Lei et al. 2006; Tolar et al. 2010; Williams et al. 1999; Zuk et al. 2001; Gronthos et al. 2011). MSCs express molecules of the major histocompatibility complex (MHC) class I at a low level and do not express molecules of MHC class II, hence they may be used in allogeneic transplantation (Prockop 2009; Haider et al. 2011). They constitute a heterogeneous population of cells and are characterized by the expression of specific surface markers including CD73, CD90, and CD105 markers, while lacking the expression of CD14, CD11b, CD79 and Cd19, CD34 and CD45 hemopoietic stem cell–specific markers, as well as CD31 endothelial markers (Lv et al. 2014; Haider 2018). Besides surface marker expression, they show specific adherence to the plastic surface and possess trilineage differentiation potential to adopt adipocyte, osteoblast, and chondrocyte phenotypes (Caplan and Correa 2011; Wang et al. 2018). This criterion of characterization has been set forth by the International Society of Cell Therapy (ISCT) which has significantly helped in harmonizing the nomenclature and biological characterization of the cell preparations being used in the experimental and clinical studies. Their autologous availability and robust nature, therefore, can be genetically manipulated to delivery genes of interest to the target organ for angiomyogenic repair as well as to enhance their therapeutic potential (Jiang et al. 2006; Haider et al. 2008) and reprogramming in to pluripotency (Buccini et al. 2012). They have also been combined with other stem cell types for combinatorial cell therapy approach (Hosseini et al. 2018).

Mechanisms of MSCs' Action

The first and the primary mechanism of MSCs' action is their transdifferentiation into morphofunctionally competent cell types and achieve the phenotype of interest,

which allows the replacement of damaged cells and contribute to the repair and restoration of damaged tissues (cartilage, bones, etc.). The second mechanism of MSCs' action is associated with the ability of MSCs to move to the sites of damage and inflammation, and secrete cytokines and growth factors, and lipid vesicles rich in bioactive cargo of proteins, lipids, and RNA as part of their paracrine activity to reduce inflammation and restore the damaged tissues (Caplan and Correa 2011; Caplan 2016; Bernardo and Fibbe 2013) (Fig. 1). Besides the abovementioned two primary mechanisms, MSCs also have immunosuppressive and anti-inflammatory effects via the suppression of proliferation and differentiation of T cells (CD4+ and CD8+ lymphocytes), reducing the activity of NK cells and activating T regulatory cells. In addition, MSCs reduce the secretion of pro-inflammatory cytokines (IL-1β, IL-6, TNFα, and IFN-γ) and boost the secretion of anti-inflammatory IL-4 and IL-10 (Spaggiari et al. 2008; Ghannam et al. 2010). More recently, MSCs surface markers including PDL1, Gal-9, CXCR4 etc., have been implicated as part of the immunosuppressive activity of MSCs (Siyu et al. 2020). Concomitantly, proangiogenic activity of MSCs induces neovascularization regionally at the site of engraftment to restore regional blood flow (Maacha et al. 2020), while apoptosis and oxidative stress are inhibited (Terai and Tsuchiya 2017). Put together, the mechanism of MSCs' therapeutic benefits is multifactorial as summarized in Fig. 1.

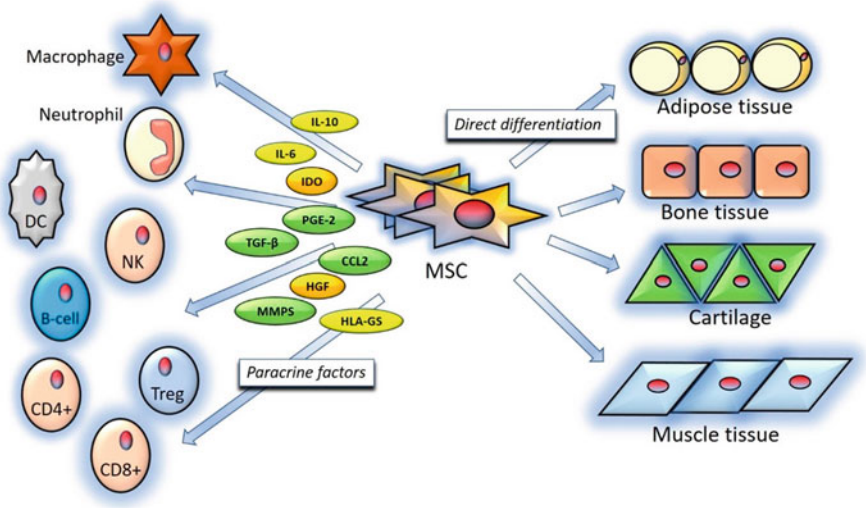

Fig. 1 General MSCs' effects grouped by the two fundamental mechanisms: (1) direct transdifferentiation of the transplanted MSCs (into cells of adipose, bone, cartilage, and muscle tissues) to replace damaged cells and (2) induction of cytokines secreted by MSCs as a part of their paracrine activity into the inflammatory medium, affecting the recipient's immune system. **Abbreviations**: (IL-6: Interleukin-6; PGE2: Prostaglandin E2; TGF-β: β-transforming growth factor; IDO: Indoleamine-2,3-dioxygenase; CCL-2: C-C-chemokine ligand 2; IL-10: Interleukin −10; HGF: Hepatocyte growth factor; MMP: Matrix metalloproteinases; HLA-G: Human leukocyte antigen-G)

Preclinical Studies

Yabana et al. demonstrated that in rats with dextran sodium sulfate (DSS)-induced colitis, MSCs, administered intravenously to the animals, migrated to the lamina propria of the damaged colon, where they activated the expression of alpha-smooth muscle actin (α-SMA), which facilitated the restoration of the epithelium (Yabana et al. 2009). It was also shown that MSCs participated in sustaining the epithelial barrier function by the repeated assembly of claudins, apical proteins of tight junctions.

The most critical role in the IBD pathogenesis is evidently played by enhanced proliferation and defective apoptosis of immune cells, which is likely related to the imbalance of Bcl-2 and Bax, essential proteins controlling apoptosis (Dias et al. 2014).

Akiyama et al. showed that systemic infusion of bone marrow–derived MSCs (BM-MSCs) induced apoptosis of T cells via the Fas-ligand (FasL)-dependent pathway and could improve the disease course in experimental murine DSS-induced colitis (Akiyama et al. 2012). However, FasL/MSCs did not induce apoptosis of recipients' T -cells and could not positively influence the colitis course. It was shown that Fas-regulated secretion of MCP-1 protein by BM-MSCs recruited T cells for FasL-mediated apoptosis. Apoptosis of T cells, in turn, leads to the induction of macrophages producing a high level of TGFβ. This results in an increased number of T-regulatory cells and, finally, in the immune tolerance of the organism.

IBD is also associated with the imbalance in subpopulations of T cells. As a result, the pro-inflammatory cytokines level grows: in CD – due to differentiation of Th1 and Th17 cells, and in UC – due to differentiation of Th2 cells. In contrast, the level of T-regulatory (Treg) cells is depressed in the peripheral blood of IBD patients (Sisakhtnezhad et al. 2017). Among Treg cells, the crucial role in the immune system suppression and sustaining the tolerance belongs to CD4+CD25+FoxP3+ cells (Akiyama et al. 2012).

Chen et al. demonstrated that intravenous MSCs administration significantly reduced the clinical severity of murine UC (weight loss, diarrhea, and inflammation) induced by trinitrobenzene sulfonic acid (TNBS) and enhanced the survival of animals (Chen et al. 2013). It was shown that MSCs reached the damaged colon and facilitated the proliferation of intestinal epithelial cells and differentiation of intestinal stem cells (determined by detecting Lgr5+-cells). Furthermore, it was mediated by suppressing both Th1 and Th17 cell–induced autoimmune and inflammatory reactions (IL-2, TNF-α, IFN-γ, T-bet; IL-6, IL-17, RORγt), as well as by enhanced activity of Th2 cells (IL-4, IL-10, and GATA-3). Besides, it was shown that MSCs induced activated CD4+CD25+Foxp3+ T-regulatory cells (TGF-β, IL-10, Foxp3).

Macrophages, dendritic cells, and B cells, known as antigen-presenting cells (APC), are also involved in the IBD pathogenesis due to their specialization in presenting an antigen to T cells and the subsequent generation of the T cell response. Macrophages play a critical role in sustaining normal intestinal homeostasis, but they

may also participate in the IBD pathogenesis (Han et al. 2021). In a healthy colon, resident macrophages exhibit an M2 phenotype, while inflammatory M1 macrophages dominate in the inflamed intestinal mucosa. In this regard, changing the balance of macrophage population to the M2 phenotype is being adopted as a novel approach in IBD therapy (Ahluwalia et al. 2018). Numerous preclinical studies have shown that MSCs can induce immunomodulating macrophages and macrophages mediate their therapeutic efficiency in experimental UC with an M2-like phenotype (Hidalgo-Garcia et al. 2018).

Jo et al. cocultured immature dendritic cells and lipopolysaccharide (LPS)-treated mature dendritic cells with MSCs for 48 h, and then analyzed the profiles of surface markers and cytokines and the regulatory role of those DC for primary splenocytes (Jo et al. 2018). Besides, the therapeutic effects of MSCs and DC cocultured with MSCs were compared for UC-affected mice. The authors demonstrated that following the coculture of MSCs with immature dendritic cells (MSCs-DC) or LPS-treated mature dendritic cells (LPS + MSCs-DC), the expression of CD11c, CD80, CD86, IL-6, TNF-α, and IFN-γ was significantly decreased. In contrast, the expression of CD11b, IL-10, and TGF-β was elevated. Besides, MSCs-DC and LPS + MSCs-DC induced CD4, CD25, and Foxp3 in primary mice-derived splenocytes. In mice with DSS-induced UC, MSCs and MSCs-DC increased the length of the colon, body weight, and survival, and caused a histological improvement. Moreover, in the MSCs and MSCs-DC groups, the expression of IL-6, TNF-α, and IFN-γ in the colon tissues was also inhibited, while the expression of IL-10, TGF-β, and Foxp3 was elevated. These data assumed that MSCs stimulate differentiation of dendritic cells into regulatory dendritic cells leading to improved chronic colitis therapy.

It was also shown that administration of MSCs could suppress activation and proliferation of B cells secreting IgG and, oppositely, stimulate the formation of CD5 + regulatory B cells (Bregs) producing IL-10. Besides, it was shown that MSCs could depress the proliferation of NK cells secreting pro-inflammatory cytokines (Liu et al. 2020).

MSCs-Derived Exosomes for Experimental IBD Therapy

MSCs-derived exosomes – extracellular vesicles obtained from MSCs – contain a large number of essential factors (Haider and Aramini 2020). In intercellular communication, exosomes are identified as efficient carriers for nucleic acids, functional proteins, lipids, mRNA, and microRNA (Samoylova et al. 2017). Thus, MSCs-derived exosomes, similar to MSCs themselves, have a potent physiological action affecting the damaged tissue repair (Zhao et al. 2019; Haider and Aramini 2020). At the same time, exosomes are more stable than MSCs and in principle are nonimmunogenic.

It was demonstrated earlier by several research groups that exosomes secreted by MSCs had a pronounced regenerative effect in the therapy of many diseases causing tissue damage, including IBD (Mianehsaz et al. 2019; Mendt et al. 2019; Mao et al. 2017). For instance, Mao et al. showed that exosomes released from human

umbilical cord–derived MSCs (hUC-MSCs) positively influenced the treatment of DSS-induced colitis and studied the primary mechanism of their effect (Mao et al. 2017). Similarly, exosomes labeled with indocyanine green (ICG) reached the colon tissue of IBD-affected mice 12 h after the injection. The IL-10 gene expression was increased, while the expression of TNF-α, IL-1β, IL-6, iNOS, and IL-7 genes was decreased in the colon and spleen tissues of mice treated with MSCs-derived exosomes. Besides, macrophages infiltration in the colon tissues was significantly reduced. It was also shown that coculturing in vitro with exosomes suppressed the expression of iNOS and IL-7 in macrophages isolated from the peritoneal cavity of normal mice. In addition, the researchers found that IL-7 expression in the colon tissue was higher for colitis patients than healthy participants of the control group. In general, the data obtained have demonstrated a potent effect of hUC-MSCs-derived exosomes on the relief of DSS-induced experimental IBD. The observed effects may be mechanistically mediated via the modulation of IL-7 expression in macrophages at molecular levels.

In a study by Yang et al., exosomes derived from MSCs preconditioned with IFN-γ were transplanted in an experimental mice model of DSS-induced colitis that essentially improved the index of activity and histological assessment of colitis, as well as reduced the fraction of Th17 cells and augmented the fraction of Treg cells (Yang et al. 2020). Molecular studies revealed that the administration of exosomes markedly inhibited the expression of Stat3 and p-Stat3, suppressing differentiation of Th17 cells. Interestingly, treatment with exosomes derived from MSCs preconditioned with IFN-γ showed the highest inhibition. Furthermore, the preliminary treatment with IFN-γ increased the level of miR-125a and miR-125b in MSCs-derived exosomes, which directly targeted Stat3, suppressing differentiation of Th17 cells. Moreover, concomitant infusion of miR-125a and miR-125b also demonstrated a therapeutic effect in colitis, accompanied by a simultaneous decrease in the Th17 cell fraction. In general, this study demonstrated that the IFN-γ treatment enhanced the efficiency of MSCs-derived exosomes in the relief of colitis, owing to increasing the level of miR-125a and miR-125b, which are bound to 3′-UTR of Stat3, to suppress differentiation of Th17 cells.

Clinical Studies

Completed Clinical Studies

Due to their therapeutic properties, MSCs (obtained mainly from the bone marrow and adipose tissue) have been actively used in numerous clinical trials on IBD therapy, with both local injections of cells and intravenous (systemic) infusions (Table 1).

Local MSCs Injections

Local administration of MSCs is used primarily for the therapy of fistulizing (extraluminal) form of CD (Ko et al. 2021). For example, Panes et al. have conducted a

Table 1 Results of major completed clinical trials on the MSCs therapy of IBD

No	Disease	Patients	Cells	Cell dose/delivery	Key findings	Study location
1.	Perianal CD	212	Allogeneic ASC	120×10^6 ($n = 107$) vs placebo ($n = 105$)/local injection	At week 52, a significantly larger part of patients receiving ASC reached the combined remission (56.3%, as compared to the control group with 38.6%) and the clinical remission (59.2% vs. 41.6% of the control group). In 1 year, no serious adverse events were observed	Forty-nine hospitals in Europe and Israel; NCT01541579 (ADMIRE-CD) (Panes 2018)
2.	Perianal CD	18 (45 total)	Allogeneic ASC, autologous ASC, and stromal vascular fraction (SVF)	$40–42 \times 10^6$ ASC, $6.5–15 \times 10^6$ SVF/local injection	Healing achieved: 40% of CD patients who received SVF, 66.6% of CD patients who received auto-ASCs, and 55.5% of CD patients who received allo-ASCs	University Hospital Fundación Jiménez Díaz, Madrid, Spain (Herreros 2019)
3.	Perianal CD	36	Autologous ASC	$3–6 \times 10^7$/local injection	At 24 months, complete healing was observed in 27 out of 36 patients (75.0%). No adverse events related to ASC administration were observed	University of Ulsan College of Medicine and Asan Medical Center, Seoul, Republic of Korea; NCT01011244 and NCT01314079 (Cho 2015)
4.	Perianal CD	24	Allogeneic ASC	$20–40 \times 10^6$/local injection	At week 24, 69.2% of the patients showed reduction in the number of draining fistulas, 56.3% of the patients achieved complete closure of the treated fistula, and 30% of the cases presented complete closure of all existing fistula tracts	Virgen del Rocio University Hospital, Seville, Spain (de la Portilla 2013)

5.	Perianal CD	21	Allogeneic BM-MSCs	1×10^7 ($n = 5$, group 1), 3×10^7 ($n = 5$, group 2), or 9×10^7 ($n = 5$, group 3) MSCs, or placebo ($n = 6$)/local injection	Thirteen out of fifteen patients (87%) treated with BM-MSCs were available for a long-term follow-up (4 years). No adverse events were associated with local injection of any dose of MSCs. In group 2 [$n = 4$], all fistulas were closed 4 years after BM-MSCs therapy. In group 1 [$n = 4$] 63%, and in group 3 [$n = 5$] 43% of the fistulas were closed, respectively. Pelvic MRI showed significantly smaller fistula tracts after 4 years	Leiden University Medical Center, Leiden, The Netherlands (Barnhoorn 2020)
6.	UC	70	Allogeneic hUC-MSCs	Group I ($n = 34$): I/V injection of 0.5×10^6 cells/kg, followed by intra-arterial injection of 1.5×10^7 MSCs 1 week later. Group II ($n = 36$): placebo (normal saline)	One month after therapy, 30/36 patients in group I showed a good response, and diffuse and deep ulcer formation and severe inflammatory mucosa were improved markedly. During the follow-up, the median Mayo score and histology score in group I were decreased while IBDQ scores were significantly improved compared with before treatment and group II ($P < 0.05$). Compared with	Qingdao University, Qingdao, Shandong, China; NCT01221428 (Hu 2016)

(continued)

Table 1 (continued)

No	Disease	Patients	Cells	Cell dose/delivery	Key findings	Study location
					group II, there were no evident adverse reactions after MSCs infusion in any of the patients in group I, and no chronic side effects or lingering effects appeared during the follow-up period	
7.	Luminal CD	82	Allogeneic hUC-MSCs	Group I ($n = 41$): 1×10^6 cells/kg once a week, four times in total. Group II ($n = 41$): placebo (normal saline)/I/V infusion	Twelve months after treatment, the CDAI, HBI, and corticosteroid dosage had decreased by 62.5 ± 23.2, 3.4 ± 1.2, and 4.2 ± 0.84 mg/day, respectively, in the UC-MSCs group and by 23.6 ± 12.4, 1.2 ± 0.58, and 1.2 ± 0.35 mg/day, respectively, in the control group ($p < 0.01$, $p < 0.05$, and $p < 0.05$ for UC-MSCs vs. control, respectively). Four patients developed a fever after cell infusion. No serious adverse events were observed	Shaanxi Provincial People's Hospital, Xi'an, China (Zhang 2018)
8.	UC and luminal CD	90 (69 UC and 21 CD)	Allogeneic BM-MSCs	$150–200 \times 10^6$ ($n = 39$ with UC and $n = 11$ with CD) vs control group ($n = 30$ with UC and $n = 10$ with CD)/I/V infusion	In MSCs group: statistically significant decrease in the indices of the clinical and morphological activities of an inflammatory process; clinical remission occurred in	Moscow Clinical Research Center, Moscow, Russia (Lazebnik 2010)

					40 patients (80%); discontinuing corticosteroids in 34 of the 50 patients (68%)	
9.	Luminal CD	34	Allogeneic BM-MSCs	Group 1 (15 patients): 2×10^6 MSCs/kg (at months 0, 1, and 6), with AZA 2–2.5 mg/kg. Group 2 (19 patients): 2×10^6 MSCs/kg (at months 0, 1, 6)/ IV infusion	Clinical remission (CDAI <150) was demonstrated in both groups in 12 months (average CDAI was 99.9 in group 1, 100.6 in group 2)	Moscow Clinical Research Center, Moscow, Russia (Knyazev 2018a)
10.	Perianal CD	22	Allogeneic BM-MSCs	2×10^6 MSCs/kg I/V infusion and 10–20 $\times 10^6$ local injection	According to the study results, in 12 weeks the cure of simple fistulas was noted in 8 patients (66.6%) of group I, in 6 patients (60%) of group II, and in 1 patient (7.14%) of group III. In 6 months, the simple fistulas were still healed in 8 patients (66.6%) of group I, in 6 patients (60%) of group II, and in 1 patient (7.14%) of group III. In 12 months, the healing was sustained in 7 patients (58.3%) of group I, in 6 patients (60%) of group II, and in 2 patients (14.3%) of group III. In 24 months, the closure of fistulas was sustained in 5 patients (41.6%) of group I, in 4 patients (40%) of group II, and in 0 patients (0%) in group	Moscow Clinical Research Center, Moscow, Russia (Knyazev 2018b)

(continued)

Table 1 (continued)

No	Disease	Patients	Cells	Cell dose/delivery	Key findings	Study location
					III. In conclusion, it was demonstrated that combined cell and anti-cytokine therapy of CD with perianal lesions reliably provided more frequent and prolonged closure of simple fistulas, as compared to antibiotic and immunosuppressive therapy, and reduction of the relapse incidence, as well	

Abbreviations: *CD* Crohn's disease, *UC* ulcerative colitis, *ASC* adipose tissue–derived stem cells, *SVF* stromal vascular fraction, *MSCs* mesenchymal stem cells, *BM-MSCs* bone marrow–derived mesenchymal stem cells, *hUC* human umbilical cord, *CDAI* Crohn's Disease Activity Index, *HBI* Harvey-Bradshaw Index, *AZA* azathioprine

double-blind, randomized, placebo-controlled study (ADMIRE CD Study) to establish the safety and study the long-term efficiency of a single local administration of allogeneic adipose tissue–derived MSCs (Cx601), for the treatment of CD patients with hard-to-treat draining complicated perianal fistulas (Panés et al. 2018). The study was conducted in 49 clinical centers of Europe and Israel. The trials enrolled a total of 212 patients (ClinicalTrials.gov: NCT01541579). The patients were randomly distributed (1:1) into groups, which received either a single local injection of Cx601 (120×10^6 cells) or placebo (control group) in addition to the standard care. The final indices of efficiency, estimated in the modified population of intended to treat (randomly assigned, receiving the treatment, with one or several efficiency estimations after the basic level) at week 52, including a combined remission (closure of all the treated external fistula tracts, draining at the initial level in the absence of accumulations of >2 cm, confirmed by magnetic resonance imaging; MRI) and clinical remission (absence of draining fistulas). Earlier, the same researchers reported a primary endpoint of a study at week 24 (combined remission in 51.5% patients receiving Cx601, compared to 35.6% in the control group, the difference being 15.8%; 97.5% confidence interval 0.5–31.2; $P = 0.021$) (Panés et al. 2016). At week 52, a significantly larger section of the patients receiving Cx601 reached the combined remission (56.3%, as compared to the control group with 38.6%, 17.7%difference; 95% confidence interval 4.2–31.2; P = 0.010) and the clinical remission (59.2% vs. 41.6% of the control group with the 17.6% difference; 95% confidence interval 4.1–31.1; $P = 0.013$). The safety was sustained for 52 weeks; side effects were observed in 76.7% of group Cx601 patients and 72.5% of the control group patients.

The researchers concluded that according to the results of the phase 3 study of CD patients with treatment-resistant perianal fistulas, the researchers have concluded that Cx601 is safe and efficient for closures of external fistulas, compared to placebo, in one year of the study. Based on the ADMIRE CD Study results, Darvadstrocel (Alofisel), a medication based on MSCs derived from the adipose tissue, has been developed (Scott 2018). This is the first MSCs-based cell preparation approved in the EU to treat complicated perianal fistulas in adult patients with nonactive/moderately active luminal CD when fistulas do not respond to one or more standard therapies.

Herreros et al. have published the data of a clinical study that assessed 45 patients with 52 surgically resistant anal fistulas of various etiology (of them 18 patients with perianal fistulas caused by CD) (Herreros et al. 2019). The patients' response to MSCs therapy of different types was monitored, with cells, including allogeneic MSCs from the adipose tissue (ASCs), autologous ASCs, and a stromal vascular fraction (SVF), which were believed to contain ASCs with a minimal addition of adipocytes and erythrocytes.

In 40 out of 42 cases of perianal fistulas (95.2%), either healing or improvement was shown in 6.6 weeks on average (in the observation time of 2–36 weeks). The cure occurred in 22 out of 42 cases (52.4%). Most of the patients were cured in 5.8 months on average (in the observation time of 0.5–24 months). The disease course in the 42 patients was assessed depending upon the applied cell preparations.

The degree of cure reached 13/23 (56.5%) for SVF, 3/9 (33.3%) for autologous ASCs, and 6/10 (60%) for allogeneic ASCs. The administered cell dose was also analyzed, with the average value of 43.9 million (in the range of 3–210 million cells) for the cases of cure.

If to focus on perianal fistulas caused by CD, 18/18 patients (100%) demonstrated healing or improvement/partial response, beginning from 5.3 weeks on average (in the observation time of 2–12 weeks). The cure occurred in 10/18 (55.5%) cases. Most of the patients were cured in 6.5 months (in the observation time of 0.5–24 months).

The disease course in those 18 patients was also assessed depending on the cell preparations used. The degree of cure reached 40% for SVF, 66.6% for autologous ASCs, and 55.5% for allogeneic ASCs. The mean administered dose in the cure cases was 43.9 million (in the range of 3–210 million cells). In all the cases of CD-associated perianal fistulas, the surgical technique was applied: the curettage, closing of the fistula tract, and injection of cells (Herreros et al. 2019).

The phase 2 clinical trial on the application of autologous ASCs for CD-associated perianal fistulas with a high rate of recurrence has shown their safety and therapeutic potential with a stable response for 2 years (Cho et al. 2015). In this phase 2 study, 41 patients initially participated. In 24 months, the complete cure was observed in 27 out of 36 patients (75.0%) (the data from 5 patients were absent in 24 months). No ASCs-based treatment-related adverse effects were observed. Moreover, the complete closure of the fistula was stable after the initial treatment. These results also testified that autologous ASCs were efficient in the treatment of CD-associated fistulas.

De la Portilla et al. have conducted an open-label, single-arm clinical trial which included 24 CD patients with perianal fistulas from six hospitals in Spain (de la Portilla et al. 2013). Twenty million ASCs were administered locally in one draining fistula tract. At week 12, if the fistula had not completely closed, 40 million more ASCs were administered. The patients were monitored up to week 24 after the first treatment.

During 6 months of follow-up, no serious adverse events were observed, attesting the treatment as sufficiently safe. At week 24, the number of fistulas was reduced in 69.2% of patients, the complete closure of treated fistulas was observed in 56.3% of the patients, and in 30% of the cases all the fistulas were completely closed. The criteria used to grade the extent of closure were the following: absence of draining and complete re-epithelization, and the MRI-confirmed absence of accumulations. The MRI Score of Severity showed a noticeable reduction at week 24. Thus, the applied ASCs-based therapy appeared safe and fairly efficient for CD-associated perianal fistulas.

A double-blind dose-finding study on the allogeneic BM-MSCs treatment of refractory perianal fistulizing CD was conducted at Leiden University Medical Center in 2012–2014 (Barnhoorn et al. 2020). The study involved 21 patients; three regimes of local MSCs administrations were applied: cohort 1 – five patients, 1×10^7 cells, cohort 2 – five patients, 3×10^7 cells, and cohort 3 – five patients, 9×10^7 cells. The patients were assessed for 4 years, with the registration of

clinical events, monitoring the fistula closure, and measuring the level of anti-HLA antibodies, pelvic MRI, and rectoscopy.

The long-term follow-up was performed in 13 patients (four from cohort 1, four from cohort 2, and five from cohort 3). No serious side effects of the therapy were observed. In two patients, malignancies were observed; however, these were reported as unrelated to the cell-based therapy. During 4 years of follow-up, the closure of fistulas was observed in all the cohort 2 patients, in 63% of cohort 1 patients, and in 43% of cohort 3 patients. No anti-HLA antibodies were detected in 24 weeks and 4 years of posttreatment follow-up. The fistula tracts became notably smaller, according to the MRI data. This study demonstrated that local application of BM-MSCs was safe and efficient for fistula closures.

A promising variation of the MSCs treatment for perianal fistulas is the use of a bioabsorbable matrix as a carrier for the cells. A Gore BioA Fistula Plug based on a bioabsorbable material was earlier tried in a multicenter study of high anal fistulas, including those in CD patients (Ommer et al. 2012). The study showed a rather high efficiency of such plugs in the treatment of fistulas; in particular, two out of four study participants with CD had complete healing in 6 months. Another development of the plug technique was its combined use with MSCs. A six-months-long study at Mayo Clinic (ClinicalTrials.gov Identifier: NCT01915927), including 12 patients, was dedicated to the treatment of fistulas with autologous ASCs deposited onto a Gore BioA Fistula Plug (Dietz et al. 2017). ASCs were harvested from the patients for autologous transplantation, and 6 weeks later the fistula plug loaded with autologous ASCs (named MSCs-MATRIX) was placed during a surgical intervention. Before the surgical procedure, ASCs were thawed and cultured on a Gore Bio-A Fistula Plug for 3–6 days in a polypropylene bioreactor. Each plug contained about 20×10^6 cells.

The primary study objective was to establish the safety and efficacy of autologous MSCs-MATRIX in the treatment of recurrent anal fistulas. The criteria for the secondary endpoint of the study were both clinical and radiographical. The former included: (1) partial response, when the drainage and symptoms reported by a patient were notably reduced, and (2) complete healing, when the drainage was not seen either without any action or with a mild pressure in 6 months after the treatment. The latter criterion included the narrowing and shortening of the fistula tract, as well as the absence of an abscess, as visualized by MRI (T2-weighted hyperintense fistula tract on a T2-weighted fast spin echo image). Quantitatively, the MRI results were presented in percent difference from the baseline and using the Van Assche perianal fistula severity score.

The applied MSCs-MATRIX plug for a fistula did not cause any serious effects during the 6 months of observation. Ten of the twelve patients (83%) in 6 months had clinical and radiographic signs of the complete healing. Thus, the bioabsorbable plugs containing MSCs proved themselves safe and efficient for chronic perianal fistulas.

A recently published systematic review and meta-analysis by Cao et al. have estimated the efficiency of stem cells (MSCs derived from the bone marrow and adipose tissue) in the treatment of CD-associated fistulas of any form (Cao et al. 2021).

In total, a total of 29 clinical studies involving 1252 patients were included in the review and analyzed. It was shown that the group of patients with CD-associated fistulas, to whom stem cells were transplanted, demonstrated a higher degree of fistula healing as compared to the placebo-treatment group (61.75% vs. 40.46%, or 2.21, 95% CI 1.19–4.11, $P < 0.05$). The group of patients who received stem cells in the dose of 3×10^7 cells/mL had a 71.0% acceleration of fistula healing vs. the groups of stem cell treatment with other doses (RR 1.3, 95% CI 0.76–2.22). The percentage of cured patients with perianal and trans-sphincteric fistulas was higher than patients with rectovaginal fistulas (77.95% vs. 76.41%). It is of interest that Crohn's Disease Activity Index (CDAI) and Perianal Disease Activity Index (PDAI) temporarily increased 1 month after stem cell–based therapy; however, they returned to the initial level 3 months after the treatment. Moreover, the incidence of side effects related to the treatment was significantly lower in the MSCs-treated group than in the placebo-treatment group (RR 0.58, 95% CI 0.30–1.14). The conducted study has shown that the application of stem cells, especially ASC, is a promising approach in the treatment of CD-associated fistulas, based on its higher efficiency and lower incidence of adverse events.

Intravenous MSCs Administration

Systemic (intravenous) administration of MSCs is used mainly in the therapy of luminal (inflammatory) forms of IBD (Ko et al. 2021).

In a randomized placebo-controlled clinical trial (ClinicalTrials.gov: NCT01221428), Hu et al. studied the safety and efficiency of hUC-MSCs in treating moderate and severe UC (Hu et al. 2016). Thirty-four UC patients were included in group I and received an MSCs infusion in addition to the basic treatment, while 36 patients in group II received saline in addition to the basic treatment. One-month post-treatment, the incidence of diffuse and deep ulcers and severe inflammatory processes in the mucosa was essentially reduced in 30 patients of group I. During the following observation, the average score of the Mayo scale and the histological score were decreased in group I, while the IBDQ score was significantly improved as compared to before the treatment and group II ($P < 0.05$). Furthermore, in comparison with group II, no apparent adverse reactions were observed after MSCs infusion in group I patients. Again, no chronic or long-lasting side effects were observed during the entire observation period. Thus the authors demonstrated that MSCs infusion was a safe and efficient strategy to treat UC.

Zhang et al. studied the safety and efficiency of hUC-MSCs to treat CD (Zhang et al. 2018). Eighty-two patients with diagnosed CD who had received the supporting steroid therapy for more than 6 months were included in the study. Forty-one patients were randomly assigned for administering four peripheral intravenous infusions of 1×10^6 hUC-MSCs/kg, one infusion per week. The patients were observed in the dynamics for up to 12 months. CDAI, Harvey-Bradshaw Index (HBI), and the dosage of corticosteroids were evaluated. As a result of the treatment, CDAI, HBI, and the dosage of corticosteroids decreased by 62.5 ± 23.2, 3.4 ± 1.2, and 4.2 ± 0.84 mg/day, respectively, in the hUC-MSCs group, and by 23.6 ± 12.4, 1.2 ± 0.58, and 1.2 ± 0.35 mg/day, respectively, as compared to the control group

($p < 0.01$, $p < 0.05$, and $p < 0.05$ for the hUC-MSCs group vs. the control, respectively). Four patients developed fever after the cell-based infusion. No serious adverse events were observed. The researchers concluded that hUC-MSCs were efficient in CD treatment, though occasionally may cause mild side effects.

In one of our studies, 22 patients with exacerbation of moderate and severe UC were treated with allogeneic BM-MSCs (Knyazev et al. 2016). The patients were divided into two groups. Patients of group I ($n = 12$), in addition to the standard anti-inflammatory therapy, received MSCs according to the following protocol: 0 (first infusion), week 1, and week 26, followed by every 6 months for the subsequent years of observation. Patients of group 2 ($n = 10$) received the standard anti-inflammatory therapy with 5-aminosalicylic acid (5-ASA) and glucocorticosteroids (GCS). Of group I patients, 58.3% had a severe UC exacerbation, and 41.7% had moderate UC exacerbation; in group II, the severe and moderate UC patients constituted 60% and 40%, respectively. Total colitis was established in 33.3% of group I patients and in 40% of group II patients; left-sided colitis was observed in 66.7% and 60% patients, respectively. The efficiency criterion for the therapy was a no-relapse course of the disease for 12 months. The UC clinical activity was estimated by the Rahmilevich score, endoscopic activity – by the Mayo score. The control over the dynamics of clinical, laboratory, and endoscopic indices was performed in 2, 6, and 12 months, then yearly for 3 years. During the first year of observations, in group I, a UC relapse occurred in two patients (16.7%), in group II – in three patients (30%). The relative risk (RR) was 0.3 (95% CI 0.08–1.36; $p = 0.2$; $\chi^2 = 1.47$). The Rahmilevich Clinical Activity Index was 3.33 ± 0.54 points in group I, 4.4 ± 1.13 points in group 2 ($p = 0.81$), the Mayo score was 3.1 ± 0.85 and 3.9 ± 1.06, respectively ($p = 0.66$). In 2 years of observation, the risk of a UC relapse in group I was three times lower than that in group 2 ($p = 0.03$). The average duration of remission in group I was 22 months, in group II – 17 months ($p = 0.049$). In 3 years of observation, the duration of remission was 22 and 20 months, respectively ($p = 0.66$). The Rahmilevich Clinical Activity Index was 4.75 ± 1.13 points in group I, 8.1 ± 1.1 points in group II ($p = 0.001$). In conclusion, the study reliably demonstrated that MSCs infusions enhanced the efficiency of anti-inflammatory therapy in patients with the acute UC.

In another study (Lazebnik et al. 2010), we used intravenous administration of allogeneic BM-MSCs to treat 39 UC patients and 11 CD patients (with the control groups of 30 UC patients and 10 CD patients). A statistically significant decrease in the indices of the clinical and morphological activities of the inflammatory process was noted after the MSCs transplantation in 39 patients with UC and in 11 patients with CD as compared to the control groups. A clinico-morphological remission occurred in 40 patients (80%). In addition, the use of MSCs made it possible to discontinue GCS in 34 out of 50 patients (68%) with the hormone-dependent and hormone-resistant forms of UC and CD and reduced the dose of prednisolone to 5 mg/day in 7 patients, with administering 5-ASA only.

Our later study estimated the efficiency of BM-MSCs therapy of CD patients receiving azathioprine (AZA) (Knyazev 2018a). The study included 34 patients with the inflammatory (luminal) CD form. Group I ($n = 15$) received an anti-inflammatory

therapy with the use of MSCs culture in combination with AZA. Group II ($n = 19$) received MSCs without AZA. The severity of attack was estimated in CDAI points. The blood serum was studied, including immunoglobulins (IgA, IgG, IgM), interleukins (IL) 1β, 4, and 10, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), transforming growth factor-1β (TGF-1β), C-reactive protein (CRP), thrombocytes, and erythrocyte sedimentation rate (ESR) in 2, 6, and 12 months from the MSCs therapy beginning. The initial mean CDAI was 337.6 ± 17.1 points in group I and 332.7 ± 11.0 points in group II ($p = 0.3$). In both groups, a significant decrease in CDAI was noted in two and 6 months from the therapy beginning: in 2 months – to 118.9 ± 12.4 in group I and to 120.3 ± 14.1 in group II ($p = 0.7$), in 6 months – to 110.3 ± 11.1 in group I and to 114.3 ± 11.8 in group II ($p = 0.8$). In 12 months, the CDAI was 99.9 ± 10.8 in group I and 100.6 ± 12.1 in group II ($p = 0.8$); in 24 months – 133.2 ± 28.3 in group I and 120.8 ± 15.5 in group II ($p = 0.2$); in 36 months – 139.9 ± 23.4 and 141.7 ± 20.8 ($p = 0.9$) in group I and II, respectively. The IgA, IgG, and IgM levels were significantly lower in the group of patients with a more extended history of the disease and prolonged use of AZA. After the MSCs infusions, in both groups, we observed a tendency to the increase in the pro- and anti-inflammatory cytokines, with a significantly lower level of pro-inflammatory cytokines (INF-γ, TNF-α, and IL-1β) in group I. The latter indicates potentiation of the immunosuppressive effect of MSCs and AZA, which provides a more pronounced anti-inflammatory effect. Moreover, it has been demonstrated that MSCs transplantation stimulates elevation of the initially reduced concentration of immunoglobulins and cytokines in the blood serum and restoring their balance with the setting-in of the clinical remission.

Interesting results were obtained when comparing the effects of combined (local and systemic) administration of BM-MSCs, anti-cytokine therapy (infliximab, IFL) and antibiotic, and immunosuppressive therapy on the healing of CD-associated simple perianal fistulas (Knyazev 2018b). The first group of CD patients aged from 19–58 years (Me – 29; $n = 12$) received MSCs systemically according to a scheme and locally. The second group aged from 20–68 years (Me – 36, $n = 10$) received IFL according to a scheme. The third group aged from 20–62 years (Me – 28, $n = 14$) received antibiotics and immunosuppressants. According to the study results, in 12 weeks the cure of simple fistulas was noted in eight patients (66.6%) of group I, in six patients (60%) of group II, and in one patient (7.14%) of group III. In 6 months, the simple fistulas were still healed in eight patients (66.6%) of group I, in six patients (60%) of group II, and in one patient (7.14%) of group III. In 12 months, the healing was sustained in seven patients (58.3%) of group I, in six patients (60%) of group II, and in two patients (14.3%) of group III. During 24 months follow-up, the closure of fistulas was sustained in five patients (41.6%) of group I, in four patients (40%) of group II, and no patient (0%) in group III. In conclusion, it was demonstrated that combined cell and anti-cytokine therapy of CD with perianal lesions reliably provided more frequent, sustained, and prolonged closure of simple fistulas, as compared to antibiotic and immunosuppressive therapy, and reduction of the relapse incidence as well (Fig. 2).

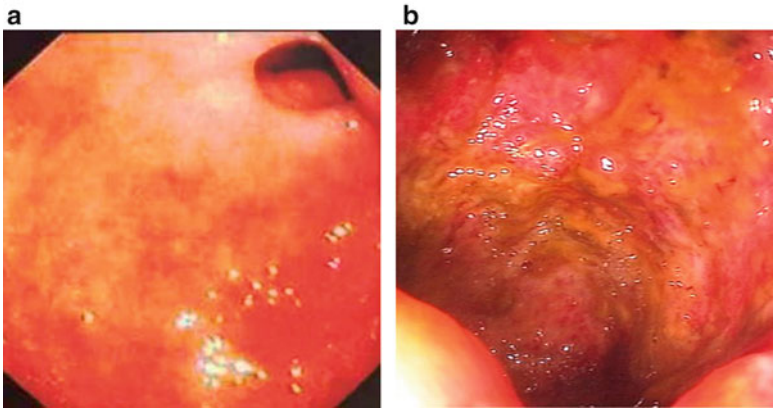

Fig. 2 Colonoscopy of a 38-year-old female patient with CD, before and after MSCs-based cell therapy [50]. (**a**) The internal opening of the fistulous tract in the lower part of the rectum ampulla before the treatment; (**b**) Twelve weeks post-treatment, healed fistula

In their recent publication, Ko et al. have provided an extensive analysis of the safety and efficiency of MSCs-based cell therapy of IBD involving on 24 studies, in 17 of which MSCs were administered locally while in the remaining 7 studies MSCs were administered systemically (Ko et al. 2021). The authors concluded that local MSCs injection-based protocol for fistulizing (extra-luminal) CD form demonstrated long-term efficiency, with the good safety level. However, regarding the efficiency of systemic MSCs infusion, the evidence was ambiguous, in the authors' opinion. They noted the marked methodological heterogeneity of the studies (first of all, due to different MSCs sources), along with the absence of facts confirming that MSCs reach the colon after an intravenous injection, and found that the safety profile was not always clearly demonstrated. At the same time, in our studies mentioned above, unequivocal pieces of evidence have been obtained for the efficiency of systemic allogeneic MSCs infusions in the IBD therapy (Lazebnik et al. 2010; Knyazev et al. 2016; Knyazev et al. 2018a, b).

In a larger and a more extensive study with a 5-year follow-up, we compared the safety profile of BM-MSCs and a standard treatment using 5-ASA, GCS, and immunosuppressive agents (Knyazev et al. 2015). The study included 103 IBD patients (56 UC patients and 47 CD patients) who received the MSCs therapy and 208 patients receiving the standard anti-inflammatory therapy (but not anti-TNF therapy). All the participants were similar in their demographic characteristics and disease features. No differences were found in the development of acute posttransfusion toxicity, infectious complications, exacerbation of chronic inflammatory diseases, serious infectious complications, malignancy, and death, with the exception of fever in some patients treated with MSCs. Thus, cell-based therapy was considered safe for the clinical practice.

Proceeding Clinical Studies

Currently (by March 2021), 14 proceeding clinical trials involving MSCs-based cell therapy for the IBD treatment have been registered at Clinicaltrials.gov (Table 2). Included in these clinical studies are autologous MSCs-based cell therapy (two studies) and allogeneic MSCs-based cell therapy (12 studies). BM-MSCs are used in seven studies, MSCs derived from the adipose tissue will be used in five studies, one study will use MSCs derived from the umbilical cord blood, while one study will use Wharton's jelly–derived cells. Ten clinical trials are dedicated to the treatment of CD, while the other four trials will focus UC treatment. Local MSCs administration protocol will be used in 12 studies while systemic administration will be used in the other two studies.

The mentioned above Mayo Clinic study of MSCs-impregnated plugs for perianal fistulas (Dietz et al. 2017) has a very promising development with young patients (Pediatric MSCs-AFP Sub-Study for Crohn's Fistula, NCT03449069). A single dose of 20 million autologous MSCs is suggested to use in five pediatric patients aged from 12–17 with CD-associated perianal fistulas. The treatment will begin with a standard therapy of infection drainage and placement of a draining seton. In 6 weeks after the placement of a draining seton, it will be removed and replaced with a fistula plug (MSCs-coated Gore Bio-A Fistula Plug). The follow-up period will be 24 months, with the treatment safety and the fistula response being monitored.

Table 2 The ongoing clinical trials on the MSCs therapy of IBD (according to clinicaltrials.gov by March, 2021)

No.	Title, ClinicalTrials.gov ID	Disease	Cell type and source	Delivery	Location
1.	Use of Mesenchymal Stem Cells in Inflammatory Bowel Disease; NCT03299413	UC	Allogeneic MSCs, Wharton's jelly	Intravenous	Cell Therapy Center, Amman, Jordan
2.	Angiographic Delivery of AD-MSCs for Ulcerative Colitis; NCT04312113	UC	Autologous ASC	Intraarterial	Mayo Clinic in Rochester, Minnesota, USA
3.	Adipose Mesenchymal Stem Cells (AMSCs) for Treatment of Ulcerative Colitis (AMSCs_UC); NCT03609905	UC	Allogeneic ASC	Local	Liaocheng City People's Hospital, Liaocheng, Shandong, China
4.	Study of Mesenchymal Stem Cells for the Treatment of Medically Refractory Ulcerative Colitis (UC); NCT04543994	UC	Remestemcel-L (Allogeneic BM-MSCs)	Local	Cleveland Clinic, Cleveland, Ohio, USA

(continued)

Table 2 (continued)

No.	Title, ClinicalTrials.gov ID	Disease	Cell type and source	Delivery	Location
5.	Mesenchymal Stem Cells for the Treatment of Perianal Fistulizing Crohn's Disease; NCT04519671	CD	Allogeneic BM-MSCs	Local	Cleveland Clinic, Cleveland, Ohio, USA
6.	Study of Mesenchymal Stem Cells for the Treatment of Ileal Pouch Fistula's in Participants with Crohn's Disease (IPAAF); NCT04519684	CD	Allogeneic BM-MSCs	Local	Cleveland Clinic, Cleveland, Ohio, USA
7.	Mesenchymal Stem Cells for the Treatment of Rectovaginal Fistulas in Participants with Crohn's Disease; NCT04519697	CD	Allogeneic BM-MSCs	Local	Cleveland Clinic, Cleveland, Ohio, USA
8.	Mesenchymal Stem Cells for the Treatment of Pouch Fistulas in Crohn's Disease; NCT04073472	CD	Allogeneic BM-MSCs	Local	Cleveland Clinic, Cleveland, Ohio, USA
9.	Study of Mesenchymal Stem Cells for the Treatment of Medically Refractory Crohn's Colitis; NCT04548583	CD	Allogeneic BM-MSCs	Local	Cleveland Clinic, Cleveland, Ohio, USA
10.	A Follow-Up Study to Evaluate the Safety of ALLO-ASC-CD in ALLO-ASC-CD-101 Clinical Trial; NCT03183661	CD	Allogeneic ASC	Local	Anterogen Co., Ltd., Seoul, Republic of Korea
11.	MSCs Intratissular Injection in Crohn's Disease Patients; NCT03901235	CD	Allogeneic BM-MSCs	Local	CHU de Liège, Liège, Belgium
12.	A Study to Evaluate the Safety of ALLO-ASC-CD for	CD	Allogeneic ASC	Local	Yonsei University College of

(continued)

Table 2 (continued)

No.	Title, ClinicalTrials.gov ID	Disease	Cell type and source	Delivery	Location
	Treatment of Crohn's Disease; NCT02580617				Medicine, Seoul, Korea, Republic of
13.	Long-Term Safety and Efficacy of FURESTEM-CD Inj. in Patients with Moderately Active Crohn's Disease (CD); NCT02926300	CD	Allogeneic MSCs, UC	Local	Inje University Haeundae Paik Hospital, Busan, Korea, Republic of
					Yeungnam University Medical Center, Daegu, Korea, Republic of
					Seoul National Universty Bundang Hospital, Seongnam-si, Korea, Republic of (and 4 more. . .)
14.	Pediatric MSCs-AFP Sub-Study for Crohn's Fistula; NCT03449069	CD	MSCs-AFP (Patch coated with ASC)	Local	Mayo Clinic in Rochester, Rochester, Minnesota, USA

Abbreviations: *CD* Crohn's disease, *UC* ulcerative colitis, *ASC* adipose tissue–derived stem cells, *MSCs* mesenchymal stem cells, *BM-MSCs* bone marrow–derived mesenchymal stem cells, *hUC* human umbilical cord, *MSCs-AFP* mesenchymal stem cell–coated anal fistula plug

Conclusion

The numerous open and randomized clinical studies on MSCs in the IBD therapy have unequivocally shown the safety of this approach and its potential efficiency, including the traditional treatment-resistant cases. The therapeutic action of MSCs originates from the potent immunomodulating effect resulting in the reduction of the autoimmune inflammation and stimulation of reparative processes in the intestinal mucosa. In turn, it prolongs the duration of remission, decreases the risk of relapses, and the frequency of hospital admissions. Based on the conducted clinical trials, a first medication based on allogeneic MSCs derived from the adipose tissue, Darvadstrocel (Alofisel, Takeda), has been approved in the EU for the therapy of complicated perianal fistulas in patients with luminal CD. A promising approach in the treatment of fistulizing CD is the use of biomaterials as carriers for MSCs (fistula plugs coated with MSCs). Firstly, the donor cell survival is higher on a biomaterial. Secondly, the application of autologous MSCs enhances the therapeutic effect of fistula plugs.

However, presently there is no single established optimal protocol for MSCs transplantation in IBD therapy. Additional studies are warranted on the optimal MSCs source, dosage, delivery method, and optimal treatment frequency. Despite the achievement of positive results, further preclinical and clinical studies are

required to enhance the efficiency of both local and systemic MSCs transplantation. Along with BM-MSCs and ASC, the use of MSCs from the placenta appears promising. With the techniques enhancing the efficacy of MSCs production, such as 3D culturing and application of large-volume bioreactors, it may essentially lower the price of MSCs production and make this unique therapy available for a wide circle of patients.

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Extracellular Vesicles-Based Cell-Free Therapy for Liver Regeneration

8

Mustapha Najimi and Khawaja Husnain Haider

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Abstract

The substantial worldwide burden of liver diseases and related complications is in line with the regular developments of innovative therapeutic strategies that could alleviate the number of patients requiring liver transplantation, the gold standard of care approved so far. Cell transplantation has brought new perspectives to treat those patients while keeping their own livers. The concept was simple as the transplanted cells were used to promote parenchymal regeneration and/or repairing. Isolated hepatocytes were initially applied and demonstrate the proof of concept of this approach at the clinical level. Stem cells, second-generation advanced therapy medicinal products, have provided many technological and logistical solutions to improve the wide clinical use of cell therapy. Mesenchymal stem cells were extensively developed to this end and show a significant ability to migrate in the recipient diseased liver, to differentiate in situ, and to exhibit

M. Najimi (✉)
Laboratory of Pediatric Hepatology and Cell Therapy, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium
e-mail: mustapha.najimi@uclouvain.be

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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interesting immunomodulatory, immunosuppressive, and anti-fibrotic features. Most of those paracrine effects were mediated by potent bioactive molecules secreted by those stem cells. Extracellular vesicles represent a significant part of this secretome and display several interesting characteristics that support their development for liver-cell-free therapy. This chapter summarizes and discusses the significant advances related to cell-based and cell-free therapies currently achieved for the treatment of liver diseases. It also addresses the current challenges that extracellular vesicles-based therapy is dealing with before a future clinical use.

Keywords

Cargo · Exosomes · Extracellular vesicles · Hepatic · Liver diseases · Noncoding RNAs · Secretome · Stem cells

Abbreviations

ACLF	Acute on chronic liver failure
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
iPSC	Induced pluripotent stem cells
HSCs	Hepatic stellate cells

Introduction

The heart and the liver, together with the lungs, constitute the central trio of organs that critically manage the primary physiological activities of the human body and also control the activities of other organ systems. Any defects impacting one or the other organ can consequently be fatal.

Due to its contractile function, the heart distributes oxygenated and nutrient-rich blood to all the body tissues and organs besides preserving body temperature (Yilmaz et al. 2013). Since antiquity, much interest has been focused on heart-related research. It has been described as a hot and dry organ and a center of intelligence and vitality in the human body. The structural and functional development of the heart is critical from birth to weaning as its increased activity has to be perfectly aligned with growing locomotor activity and thermoregulation (Rakusan et al. 1984; MacLellan and Schneider 2000). Such proliferative growth will completely stop after the end of this critical period (Rumyantsev 1977; Brodsky 1990; MacLellan and Schneider 2000).

On the other hand, the liver is the largest gland in the human body and concomitantly manages a broad range of high-level physiological and complex functions such as detoxification, protein synthesis, and biochemical production, and any alteration of which significantly impacts the physiological functioning of other body organs (Gebhardt and Matz-Soja 2014). Galen A. has considered the liver the primary organ of the human body because it is the source of the veins and the

principal instrument of sanguification (Temkin and Straus 1946). Contrary to the heart, the critical period for the liver is from weaning to maturation (Wheatley 1972; Alison 1986; Brodsky 1990) primarily due to dietary changes (Wheatley 1972; Brodsky 1990; Vinogradov et al. 2001).

For many years, an established intimate interaction between both vital organs has been extensively reported as in the theoretical system of traditional Chinese and modern-day medicines (Zhang and Fang 2021). Avicenna also pointed out the functional interaction between the two vital organs in his famous book *The Canon of Medicine*.

The blood circulation between the liver and heart is primarily supporting such essential interorgan communication. Indeed, the hepatic artery and portal vein together allow 25% of cardiac output to reach the liver (Silvestre et al. 2014), while hepatic veins and the inferior vena cava are involved in the venous drainage toward the liver (Silvestre et al. 2014). Furthermore, such an interaction has also been described earlier during development (El-Hadi et al. 2020). The determination of the hepatic lineage is dependent on the pre-cardiac mesoderm. Indeed, it is well-accepted that the emerging ventral endoderm should be spatially close with the pre-cardiac splanchnic mesoderm to follow a hepatic fate (Fukuda 1979; Fukuda-Taira 1981). The invagination of the foregut will lead the ventral wall of the endoderm abutting the developing heart. The septum transversum, which gives rise to the epicardium of the heart and the diaphragm, also plays a significant role in modulating the differentiation of hepatocytes that constitute metabolically highly active cell population of the liver. The mesenchyme compartment of the septum transversum collaborates with the developing heart to modulate the hepatic lineage specification in a paracrine manner (Rossi et al. 2001).

Liver and Heart Connected and Non-connected Defects

Structural and/or functional defects of one or the other organ will seriously impact health by negatively altering the quality of life and decreasing its expectancy. Hence, scientists and clinicians have shown a great interest in consistently developing novel and improving the existing therapeutic strategies dedicated to treating any defects of organs, the heart and liver. Irrespective of the etiology of the disease, perturbation of the functionality of the cells remains the primary cause of various pathologies. Thus, replacing, repairing, and/or reactivating the functionally perturbed cells remains the primary focus of all the innovative therapies currently being developed to address the root cause of the problem and restore the normal function of these organs.

Heart diseases are also associated with partial or complete disruption of blood flow to the heart muscle (coronary heart diseases) and altered contractile function, which will also negatively impact the blood flow to other organs like the brain (cerebrovascular diseases) (Ren et al. 2021). Cardiovascular diseases are the leading cause of morbidity and death worldwide, affecting nearly 17.9 million people as reported in 2019. Indeed, acute events, including heart attack and stroke, may be induced due to perturbed blood flow toward the brain, or the heart, due to the

deposition of fatty plaques in the vessels, blood clots, or bleeding. Deaths related to heart attack and stroke represent 85% of cardiovascular diseases (WHO). Congenital or acquired structural abnormalities of the heart, including the valves or the heart muscle (rheumatic heart disease), as well as inherited disorders, are the other reported defects (Dimmeler 2011). Therefore, damaged or weakened organ will lead to heart failure that may be triggered by a heart attack or abnormally increased high blood pressure.

Cardiovascular diseases may be fatal for which no cure is available; however, they mostly remain preventable. Depending on the type and the severity of the illness, rigorous lifestyle changes, early start of proper medication according to the published guidelines, and surgery may ensure these patients have improved quality of life and live longer, with reduced hospitalization occurrences.

Akin to cardiovascular diseases, pathologies of the liver may lead to significantly altered liver functions. Such compromised liver function may happen at any age in life and may be caused by several internal and/or extraneous factors. Liver defects may be genetically inherited or acquired after viral infection, excessive alcohol consumption, and obesity. Inborn errors of liver metabolism happen as early as the neonatal stage and cause more than 300 different human diseases (Najimi, 2016). Although presenting features are specific for each genetic alteration, clinical disease manifestations include lethargy, vomiting, seizure, etc. Early diagnosis and appropriate treatment should limit critical extrahepatic impairments, including those occurring at the brain level. Therapeutic solutions applied so far include pharmacological, dietary, and surgical intervention (Najimi 2016). When conventional treatments fail to alleviate the disease symptoms, orthotropic liver transplantation (OLT) remains the gold standard and clinically most validated therapeutic approach to treat liver diseases (Wallot et al. 2002). However, donor shortage limits its widespread use worldwide (Kamath et al. 2001; Struecker et al. 2014; Najimi 2021).

Notably, there is mounting evidence in the literature that acute and chronic heart diseases might directly lead to reversible or irreversible deterioration of liver functions or vice versa (Poelzl and Auer 2015). This is well-supported by the existence of heart-liver and liver-heart axes primarily mediated by organokines specifically synthesized and secreted by the endocrine heart (16 cardiokines) and liver (Chiba et al. 2018; Jensen-Cody and Potthoff 2020; Meex and Watt 2017; Stephan and Haring 2013; Cannone et al. 2019). This means that the heart can modulate the liver metabolic functions, while heart diseases significantly affect the liver, an observation made since the nineteenth century (Komatsu et al. 2019). For example, heat failure may induce hypoxic hepatitis, and atrial fibrillation (arrhythmia) obviously induces hyper-coagulation. Heart failure, a general consequence of the cardiac pump dysfunction at the systolic (reduced contractility) or diastolic (altered relaxation and consequent ventricular filling) levels, is associated with severe hepatic congestion, which induces acute hepatocellular necrosis and raises liver enzyme blood concentrations, and direct or indirect serum bilirubin elevation.

The diseases of the liver also affect the functional status of the heart, including the role of nonalcoholic fatty liver disease in promoting the development of cardiovascular diseases (i.e., coronary artery disease, structural myocardial

alterations, and cardiac arrhythmias). Still, the pathophysiological mechanisms involved therein are not yet deciphered, although insulin resistance, visceral adiposity, subclinical generalized inflammation, dyslipidemia, and oxidative stress are potentially related pathways (Tana et al. 2019). End-stage liver diseases are also implicated in developing cirrhotic cardiomyopathies. In an animal model of liver cirrhosis, an abnormally increased expression of collagen isoforms was reported in the ventricular myocardial tissue that led to a raised cardiac stiffness and diastolic dysfunction (Glenn et al. 2011). Hence, sustained hemodynamic variations are behind both bridging fibrosis and cardiac cirrhosis. The consequent dysregulation of hepatic functions will lead to the impairment of the metabolism of cardiovascular drugs and potential, which can lead to an undesirable toxicity.

The functional liver-heart connection was also reported in genetic diseases. The best example is Alagille syndrome, a genetic disorder caused by abnormal bile ducts, in which subsequent perturbed bile flow induces significant scarring that prevents the liver from eliminating bloodstream wastes. Alagille syndrome is interestingly linked to an impaired blood flow from the heart to the lungs (pulmonic stenosis) (Tretter and McElhinney 2018).

The heart-liver interorgan connection is supported by the nature of the major risk factors of the heart disease (i.e., alcohol intake, unhealthy diet, etc.), whose processing and metabolism are managed primarily by the liver. Inversely, perturbation in the liver functionality (like raised blood lipids, obesity) will influence the quality of heart activity. Therefore, broadening our knowledge on both heart and liver physiopathology is mandatory for the design and dosage of preventive and therapeutic strategies dedicated to one or the other organ.

Status on Cell-Based Therapy for Liver Diseases

Although OLT remains the gold standard therapeutic option for treating liver diseases, the significantly increased donor shortage and the consequently enhanced mortality due to long waiting time limit its application worldwide (Kamath et al. 2001; Struecker et al. 2014; Najimi 2021). The more extended living grafts are unfortunately associated with posttransplantation complications and morbidity, including long-term exposure to high levels of immunosuppression regimens, deficits, or delays in development (Moreno and Berenguer 2006). This has prompted the development of innovative strategies that may restore liver function, especially for indications where liver transplantation is not the ultimate treatment, or at least support the patient while waiting for a graft to be transplanted. From the early experiment involving hepatocyte transplantation in a rat model of Crigler-Najjar syndrome by Groth et al. (1977), cell-based therapy has progressed a long way to involve stem cell and progenitor cell engraftment for liver diseases treatment (Sun et al. 2014). The cells are generally infused as a cell suspension via the vascular system without compromising or alteration of the structural integrity of the diseased liver (Najimi et al. 2016; Forbes et al. 2015). The portal vein system remains the optimal injection site from which the suspended cell will be delivered

(Regmi et al. 2019). The aim is to allow transplanted cells to reach the liver parenchyma, where the transplanted cells can function after engraftment as well as in a paracrine manner (Iansante et al. 2018). Cell-based therapy preserves the recipient's liver, and it may be repeatedly applied with no significant complications (Wang et al. 2019).

The proof of concept of cell-based therapy for treating liver diseases has been demonstrated by using hepatocytes isolated from cadaveric donors (Ibars et al. 2016). Ibars and colleagues at Hepatic Cell Therapy Unit, Valencia, have shared their experience of working with five adults and four children with inborn metabolic diseases and have reported hepatocyte transplantation as a safe and viable option to generate metabolically functioning hepatocyte post engraftment. The transplanted hepatocytes were shown to display an ability to migrate from the injection site, replace the dead cells in the recipient hepatic parenchyma, and function in situ leading to vital restoration of deficient metabolic defects. Those data have also been confirmed by using stem/progenitor cells of intrahepatic and extrahepatic origins that can be expanded and differentiated into hepatocyte-like cells in vitro before transplantation.

Some of the important parameters that significantly impact the efficacy and prognosis after cell-based liver therapy include the quality of the source organ from which the cells will be isolated, the quality and yield of cell suspension post-isolation and expansion, or post-cryopreservation and thawing, as well as the nature and severity of the targeted disease, etc. (Zhu et al. 2020). Understanding of these impacting factors will not only streamline, optimize, and standardize the protocols, but it will have practical value in terms of the fast-emerging concept of individualized treatment of the patients.

Experimental Animal Studies

Cell-based therapy dedicated to treating liver diseases has been firstly assessed on surgical animal models in which liver regeneration can be physiologically induced like after partial and total hepatectomy (Alwahsh et al. 2018; Forbes et al. 2015). Animal models of inherited metabolic diseases with single specific liver enzymatic defects have also been widely used. Mostly genetically modified, those animal models exhibit significant liver damage and hepatocyte structural and functional perturbations. These conditions provide a high advantage for transplanted healthy cells to survive, engraft, and proliferate in situ. The best example is the mouse model overexpressing the urokinase-type plasminogen activator gene within the liver as albumin promoter drove its expression. This experimental mouse model displays a severe hepatic injury, while transplanted hepatocytes are able to effectively reconstitute the whole liver mass (Sandgren et al. 1991). Crossed with the (SCID = Severe Combined Immunodeficient) mice , those genetically modified animal models did allow the deep study of human cells behavior after transplantation like in fumarylacetoacetate hydrolase knockout *Fah*−/−, *Rag2*−/−*Il2rg*−/− mice (Ohshita and Tateno 2017; He et al. 2010).

Animal models in which liver diseases were pharmacologically induced and mimicked did allow the evaluation of cell-based therapy efficacy in acquired defect settings, i.e., nonalcoholic steatohepatitis, fibrosis/cirrhosis, etc. (Al-wahsh et al. 2018), but also under acute liver failure conditions like by using acetaminophen (APAP). Although animal models have demonstrated the potential of transplanted healthy cells to integrate into recipient's livers and to correct hepatic defects and even animal improved rate of survival, the higher number of combinations involving animal models and types of transplanted cells is not yet supporting the establishment of a standardized application of cell-based therapy and its translation to the clinic. Accordingly, preclinical models deeply recapitulating human diseases at the cellular and molecular levels are still lacking although trials using large transgenic or genetically deficient animals, i.e., macaques and pigs, have been reported. On the same note, despite the use of stem cells from different tissue sources, there is an obvious lack of consensus among the stem cell researchers about the ideal cell choice (Gounder et al. 2017). From among the different cell types, mesenchymal stem cells have been extensively studied for cell-based therapy of liver diseases and have progressed to the clinical phase studies more than any other cell type (de Miguel et al. 2019).

Clinical Studies

Encouraging preclinical data of liver cell therapy have supported the exploration of its usefulness at the clinical level, primarily when no other therapeutic option can be applied. This also has been supported by the reduced invasiveness of cell delivery intervention, the repeatability of cell infusions, and safety posttransplantation (Najimi et al. 2016). After successfully recovering good quality and significantly high yield of clinically approved human liver cell suspensions, hepatocyte transplantation, under the proof of concept and first in man configurations, has been applied in several centers worldwide, and data from patients with different etiologies have been reported. Indeed, the durability of the effect posttransplantation as shown on several patients with inborn errors of liver metabolism was quite variable and dependent on several factors including the yield of cells infused, the type of the analyses performed, as well as the severity of the disease. The lack of appropriate clinical trials reporting deep investigations on safety and efficacy (only very few were reported so far) makes it very difficult to formulate any conclusions on the definitive clinical use of isolated hepatocytes. Furthermore, although hepatocyte transplantation did show its ability to be used at least as a bridge to transplantation, still limitations are hampering the rapid clinical development of such therapeutic option, including the significant scarcity of good-quality raw material and the inability to long-term preserve the isolated cells due to their poor ability to survive and proliferate in vitro as well as post-cryopreservation/thawing.

Recent advances in studying stem/progenitor cells have highlighted their potential in providing several solutions to the limitations encountered when isolated hepatocytes were used, such as self-renewal, plasticity, and paracrine potency. Stem/progenitor cells have been looked for and isolated from different tissues and organs at any age including the liver itself. Although with exciting preclinical results

like those reported on embryonic stem cells (ESCs) (Woo et al. 2012; Asahina et al. 2006) and FIRST MENTIONED HERE human-induced pluripotent stem cells (iPSCs) (Corbett and Duncan 2019; Sekine et al. 2020; Takeishi et al. 2020), only MSCs are considered one of the most well-studied and extensively characterized cell types currently applied for clinical evaluation on liver diseases (Luan et al. 2021; Zhang et al. 2020). Their use in the humans has also been supported by the excellent safety profile and tolerance reported in the translational experimental studies and the ongoing trials. Fifty-nine clinical studies are registered so far in which both allogeneic and autologous MSCs isolated from different tissues were used to target various acute and chronic liver defects. A recently published pooled analysis of 39 published studies involving MSCs for various liver defects has reported that compared with the conventional treatment, MSC therapy significantly improves liver function in terms of the model of end-stage liver disease score; albumin, alanine aminotransferase, and total bilirubin levels; and prothrombin time, up to 6 months after administration (Zhao et al. 2018). Interestingly, subgroup analysis showed that single injection via hepatic artery of MSCs was more effective than peripheral intravenous injections. Moreover, bone marrow-derived MSCs were more effective than umbilical cord-derived MSCs. Recent technological advances did allow knowing and learning more on the optimal conditions for banking, large-scale production and cryopreservation, stability of MSCs, and treatment methodology/approach, which would ultimately lead to provide the best-quality cell suspension to the patient.

Liver cells of mesenchymal phenotype and stem/progenitor profile have also been described in both mice and humans. While directly isolated from mouse livers, those cells were obtained after primary culture of human liver parenchymal cell suspensions and displayed variable levels of plasticity (Herrera et al. 2006; Najimi et al. 2007; El-Kehdy et al. 2016). Those cells isolated from the adult human liver are currently developed under industrial settings (GMP large-scale expansion) to be clinically tested as advanced therapy medicinal products to treat liver diseases. For instance, HepaStem® has been successfully used in patients with urea cycle defects and Crigler-Najjar syndrome in a phase I/II clinical study in Europe aiming at evaluating its safety and preliminary efficacy at 6 and 12 months post-infusion. In parallel to their safety profile, transplantation of those cells was associated with de novo urea synthesis in most urea cycle diseased patients. It also decreased bilirubin level only in some of the Crigler-Najjar patients (Smets et al. 2019). Both metabolic effects were reported at 6 months post-HepaStem® transplantation. Advances in understanding the behavior of MSCs in targeting liver diseases have also highlighted their potent paracrine features. Indeed, this has been revealed by both in vitro analyses in which the secretome of those cells was shown to contain several bioactive molecules and in vivo, in which many described positive effects were not associated with the engraftment of transplanted cells in the recipients' livers. HepaStem has been recently applied on cirrhotic patients with acute on chronic liver failure (ACLF) or with acute decompensation at risk of developing ACLF and has been shown to significantly improve the altered liver functions in parallel to systemic inflammation and survival rate of the transplanted patients (Nevens et al. 2021).

Cell-Free Therapy Approaches for the Liver

Thanks to the information recovered from advanced clinical trials, it becomes more consistent that the beneficial clinical effects of transplanted cells were more related to their paracrine potential and aligned with their reported low engraftment (Haider and Aziz 2017). Other lessons learned from those clinical trials are the cell expansion culture timing and costs, the high procoagulant activity of the transplanted cells, their entrapment in the lung tissue when peripherally infused which may reduce their potency, and the cytogenetic abnormalities that may happen during large-scale production and/or long-term culture in vitro that may render the cell tumorigenic post engraftment (Prockop et al. 2010; Nikitina et al. 2018). On the same note, maintenance of the quality of the cell preparation and avoidance of batch-to-batch variations remains one of the major hurdles to achieve optimal prognosis (Haider, 2018). Similarly, survival of the donor cells post engraftment significantly reduces the feasibility, although various strategies, such as transient immunosuppression and preconditioning of donor cells, have been adopted successfully (Haider et al. 2004, Xiao et al. 2004, Haider and Ashraf 2012).

The fast-emerging strategy of cell-free therapy using both soluble and particulate components of stem cell paracrine secretions has given encouraging data which is comparable with the cell-based therapy (Haider and Aslam 2018). Extracellular vesicles (EVs), one of the insoluble components of cellular paracrine secretion, have been considered to be a significant component of the MSCs' secretome that could be delivered precisely and that could have influential paracrine and endocrine contribution toward the intercellular communications (Raposo and Stahl 2019; Devaraj et al. 2021). Significant technological advancements have led to the isolation and purification of these nano-sized entities from the soluble factors of the cell secretome, and they have been extensively characterized for yield and contents – under different physiopathological conditions (Borgovan et al. 2019). EVs have also been proposed to discriminate between stem cell populations depending upon their tissue of origin, which will help in categorizing and characterizing them to reduce batch-to-batch variation in their preparation (Hur et al. 2020).

Accordingly, their potential use as a therapeutic alternative to cell transplantation may bring interesting solutions for easy and widespread clinical use. Indeed, infusion of EVs could be safer as many cell-related posttransplantation reactions, like procoagulant activity, thrombogenic effect, ectopic cell migration, and differentiation, might not be considered. The simple recovery and purification of the cell supernatant containing EVs will make the production process much cheaper while following the same production and storage paths as the small molecules. Furthermore, the structural aspects of the EVs are essential to protect the cargo contents for a smooth "physiological" transfer to the target cells after fusion with the cell membrane (Haider and Aramini 2020). This latter feature is quite interesting as it will allow the manipulation of their internal and/or membranous contents for targeted tissue and material delivery.

The diverse parenchymal and non-parenchymal cell composition of the liver should be perfectly aligned with a high level of coordinated intercellular

communication to ensure the complex vital functions that such organ manages (Devaraj et al. 2021). EVs secreted by liver cells may be critical tools for the establishment of a fine-tuned crosstalk between neighboring and distant cells at the physiological as well as pathological levels. The content of EVs has been reported to be involved in the alteration of acute and chronic immune-inflammatory responses associated with several liver diseases, including those chemically or virally induced. Such alteration is due to a perturbed communication between the various liver cell types and between the liver and other organs (Babuta and Szabo 2021). As shown in the normal liver, quiescent hepatic stellate cells (HSCs) can modulate parenchymal regeneration via cell migration and immune responses and facilitate tissue remodeling after damage. Therefore, the EVs of each liver cell type may adapt the content and yield of their cargo depending on the liver defect. Many consider the circulating pools of such entities as noninvasive diagnosis biomarkers and exploit them as potential targets for developing innovative treatments.

An increasing number of preclinical studies dealing with evaluating the therapeutic potential of EVs in liver diseases are noticed. Diverse strategies, using both native and modified EVs, have been investigated to target many aspects of liver defects, including inflammation (inhibition of infiltrating cells or potentiation of the intrahepatic immune response), tissue regeneration (inhibiting the injury and/or promoting hepatocyte proliferation), viral intrahepatic infection, fibrosis/cirrhosis, and liver cancer (Driscoll et al. 2021). Transplantation of EVs is associated with an improvement of liver defects. It has confirmed the multiple modes of action of these entities, including inhibition of inflammation, hepatocyte apoptosis, autophagy and HSCs activation, the fundamental cell event behind the initiation, and sustaining of liver fibrosis (Lee et al. 2021).

In acute liver failure or injury, the therapeutic effects of EVs have been studied posttransplantation in different appropriate animal models. MSCs derived from bone marrow, umbilical cord, and adipose tissue were mainly used and did show significant mitigation of inflammation, autophagy, and apoptosis in parallel to stimulation of hepatocyte proliferation and activation of the adaptive immune system (Jin et al. 2018; Jiao et al. 2019; Haga et al. 2017; Yao et al. 2019). Such modifications have significantly attenuated the levels of circulating inflammatory markers and the necrotic parenchymal areas and accordingly restored the altered liver functions. Those data have been associated with the presence of noncoding RNAs, like H19, miR-17, and miR-455-3p.

In the ischemia/reperfusion animal model, EVs from MSCs fuse with the membranes of the hepatocytes and stimulate their proliferation, which further significantly decreases transaminases levels and histopathological scores (Du et al. 2017). Transplantation of exosomes and their migration to the liver are followed by a significant inhibition of the ALF induced by D-GalN/LPS treatment in mice due to an inhibition of hepatic mononuclear cells and cell apoptosis (Chen et al. 2017). In addition, anti-fibrotic effects of EVs have been reported posttransplantation in the widely used CCl4-treated animal model as demonstrated by the inhibition of epithelial-to-mesenchymal transition, intrahepatic inflammation, and hepatocyte apoptosis (Li et al. 2013; Devaraj et al. 2021).

iPSCs have also been used to generate MSCs as they display higher survival and proliferation than their adult stem cell counterparts (La Greca et al. 2018). When transplanted in ALF animal models, EVs from those cells display the same therapeutic effects as restoration of normal transaminases levels, thanks to significant inhibition of hepatocellular necrosis and sinusoidal congestion (Povero et al., 2019; Chen et al., 2017). EVs from the iPSCs-derived MSCs effectively protect hepatocytes and stimulate their proliferation via the sphingosine-1-phosphate pathway (Du et al. 2017). In chronic liver disease settings, EVs from MSCs of adult (bone marrow, umbilical cord, adipose tissue, and amniotic fluid) or embryonic origin (ESC, iPSCs) can improve fibrosis as experimentally shown both in vitro and in vivo (Povero et al. 2019; Li et al. 2013; Rong et al. 2019; Mardpour et al. 2018). Several features have improved following EVs transplantation, including the expression of ECM-specific disorganization markers, of activated HSCs and infiltrated immune cells, and hepatocyte survival. So far, EVs-derived MSCs from the bone marrow, adipose tissue, and liver were studied under Hepatocellular carcinoma (HCC) settings both in vitro and in vivo (Weng et al. 2021; Bruno et al. 2013; Ko et al. 2015; Webber et al. 2015; Fonsato et al. 2018). The data reported the potential of those EVs to decrease the expression of HCC markers and inflammatory cytokines as well as the number of altered parenchymal cells (both by inducing apoptosis and decreasing proliferation) which leads to an improvement of liver functions.

Furthermore, an increase in the level and activity of circulating NKT cells and their recruitment to the diseased liver have been reported (Lou et al. 2015; Ko et al. 2015). Both coding and noncoding RNAs are implicated in those reported effects (Fonsato et al., 2018). This has led to assess the efficiency of EVs for which the cargo was modified to improve their migration (membrane engineering) as well as the targeted delivery (content engineering) of specific molecules related to the tissue and disease configurations (Tan et al. 2013; Lai et al. 2013; Ishiguro et al. 2020; Psaraki et al. 2021). Advances that may arise from studies using naïve EVs should help in addressing the molecules/pathways that would improve the therapeutic value of these entities. Accordingly, additional information is mandatory to know more on the appropriate EV doses to be applied; their quality, safety, potency; and durability of the effects posttransplantation, with the vision to implement their use at the clinical level.

Diagnostic and Therapeutic Role of EVs in Liver Diseases

Intercellular communication is not only crucial for maintaining liver homeostasis but is actively involved in initiating the disease and sustaining its progression. Under pathological conditions, the yield, the size, and the content of EVs are modulated depending on the disease severity (structural intra- and extra-organ alterations) and chronicity. Therefore, the circulating EVs will reach the target cells to deliver their encapsulated content after membrane fusion and/or to modulate the activity of specific signaling pathways consequently to membranous

protein-protein interactions. The detection of EVs in blood or other fluid samples and their longer half-life support their development as a very attractive diagnostic tool that could deliver important cellular and molecular information to the diseased organ (Newman et al. 2020). Although circulating noncoding RNAs like miRNAs are reported to be concentrated in EVs, the diagnostic potential of EVs in liver diseases is still not well demonstrated due to the very limited data available so far. Ongoing investigations should potentially lead to improve (i) the selection and purity of hepatic EVs derived from the normal and diseased livers mainly at the cellular level and (ii) the discovery of altered biomarkers specific to each hepatic defect and in fine to address a highly sensitive diagnosis of those different and complex liver defects. While the simplest view of one EV type-one disease is difficultly supported, one could expect several layers of complex combination as for instance, of EVs and/or specific contents. Therefore, extensive investigations are mandatory to address several still raised questions before EVs can be applied at the clinical level, as for instance, the determination of (i) the best source of EVs to be applied for each of liver disease type, (ii) the optimal production process, and (iii) the efficient dose of EVs (fresh, cryopreserved, single and/or repeated injection, etc.) to be infused for each specific liver disease indication. Multi-omics analyses may certainly help in compiling and exploiting information related to their biogenesis, cargo, and function (Chitoiu et al. 2020).

Conclusion and Future Perspectives

Although OLT remains the gold standard therapeutic option to treat liver diseases, many patients do not have access to it. Furthermore, the waiting time for a graft often increases, while long-term posttransplantation follow-up highlights significant liver graft hepatitis and fibrosis posttransplantation as reported in the pediatric population (Kelly et al. 2016). Liver cell and stem cell transplantation has been developed as an alternative to OLT or a bridge to transplantation. Several successes and achievements of cell-based therapy have been reported both at the preclinical and clinical levels. Cell-based therapy was initially proposed to provide healthy and highly functional cells that will participate in repairing and regenerating the recipient's diseased liver. Advances in manipulating the trialed stem cells and evaluating their effects post-transplantation have instead revealed their potent paracrine effects in mitigating liver inflammation, fibrosis, and cancer. Thus, cell-free therapy is positioned as an alternative therapeutic approach able to overcome many limitations reported with cell-based therapy. The effects observed with EVs for the MSCs field are equal to those reported with MSC-based therapies. Still, extensive knowledge and investigations are mandatory before this approach can be fully considered at the clinical level. Indeed, more efforts are needed to understand the EVs-based communication between same and different cells under normal and pathological conditions knowing the complex cell composition of the liver. This means that we have to learn more about the identity of the bioactive molecules involved as well as on their respective

mode(s) of action (direct and/or indirect) both at the cellular and molecular levels, knowing the heterogeneity of cells and factors causing liver defects. Once addressed, the logistical aspect is another milestone to achieve, in terms of cell material used for large-scale production, considering the heterogeneous aspect of MSC cultures, standardized characterization and quantification, and potency evaluation. At the in vivo level, much information is mandatory to determine the optimal formulation (fresh and/or cryopreserved), route of administration, and injection dose that should be aligned with an optimal safety profile of the transplanted patients.

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Current State of Stem Cell Therapy for Heart Diseases

9

Yong Sheng Tan, Qi Hao Looi, Nadiah Sulaiman, Min Hwei Ng, and Daniel Law Jia Xian

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Abstract

Heart disease is very common among older adults and is one of the main causes of death worldwide. With age, the functionality of the heart will decrease following the changes in the cardiomyocytes and cardiac tissue. Generally, the number of

Y. S. Tan
Cardiothoracic Department, Serdang Hospital, Kajang, Selangor, Malaysia

Q. H. Looi
Future Cytohealth Sdn Bhd, Kuala Lumpur, Malaysia

N. Sulaiman · M. H. Ng · D. Law Jia Xian (✉)
Centre for Tissue Engineering and Regenerative Medicine (CTERM), Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Jalan Yaacob Latif, Kuala Lumpur, Malaysia
e-mail: nadiahsulaiman@ukm.edu.my; angela@ppukm.ukm.edu.my; danieljx08@gmail.com; lawjx@ppukm.ukm.edu.my

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cardiomyocytes will decrease, the number of senescent cells will increase, the cardiac tissue will become thicker, and the contractility will diminish. Besides, heart diseases such as myocardial infarction and heart failure also will reduce the functionality of the heart. Currently, heart disease is normally treated with medication and surgery. In severe conditions, the patient will be recommended to opt for a heart transplant. However, medication and surgery cannot reverse the pathological changes in the heart, and it is very difficult to find a suitable heart for transplantation. Stem cell therapy offers a glimpse of hope to these patients as the cells can stimulate the proliferation of cardiac progenitor cells and cardiomyocytes as well as secrete the paracrine factors which modulate the tissue environment to promote regeneration. Even though stem cells, e.g., mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs), have been shown to differentiate into cardiomyocytes in vitro, however, there is a lack of evidence to prove that the transplanted cells can reconstitute the myocardium in vivo. The number of clinical trials using stem cells to treat heart disease is still very limited. Results from these trials suggested that stem cell therapy is safe and provides certain benefits to the patients. Nonetheless, there is still a long way to go for the researchers to identify the ideal cell source and therapy protocol to achieve a greater therapeutic effect.

Keywords

Cardiomyocyte · Heart diseases · Heart failure · Infarction · Myocardial · Stem cell · Therapy

List of Abbreviations

ACE	Angiotensin-converting enzyme
AF	Atrial fibrillation
AMI	Acute myocardial infarction
Ang-1	Angiopoietin-1
ARB	Angiotensin receptor blocker
ASCs	Adipose-derived stromal cells
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMMC	Bone marrow mononuclear cell
BMSCs	Bone marrow-derived mesenchymal stem cells
CAD	Coronary artery disease
CCS	Canadian Cardiovascular Society
CPCs	Cardiac progenitor cells
CSCs	Cardiac stem cells
CT	Computed tomography
DNA	Deoxyribonucleic acid
eNOS	Human endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ESC	Embryonic stem cell
HGF	Hepatocyte growth factor

HLHS	Hypoplastic left heart syndrome
I/C	Intracoronary
I/V	Intravenous
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
iPSC	Induced pluripotent stem cell
ISCT	International Society for Cell & Gene Therapy
LVAD	Left ventricular assist device
LVDd	Left ventricular end-diastolic internal diameter
LVEDV	Left ventricular end-diastolic volume
LVEF	Left ventricular ejection fraction
LVESV	Left ventricular end-systolic volume
MCP-1	Monocyte chemotactic protein-1
MI	Myocardial infarction
MLHFQ	Minnesota Living with Heart Failure Questionnaire
MNCs	Mononuclear cells
MSC	Mesenchymal stem cell
NO	Nitric oxide
NYHA	New York Heart Association
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PGF	Placental growth factor
PLLA	Poly-L-lactic acid
PSI	Perfusion score index
RA	Refractory angina
RCTs	Randomized controlled trials
ROS	Reactive oxygen species
SAQ	Seattle Angina Questionnaire
SPECT	Single-photon emission computed tomography
T/E	Transendocardial
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
UC-MSCs	Umbilical cord-derived mesenchymal stem cells
VEGF	Vascular endothelial growth factor
WMSI	Left ventricular-wall motion score index

Introduction

A lively heart is vital to keep the body healthy and to maintain the body's homeostasis. The heart, together with the vascular system, is responsible for delivering blood throughout the body. Nowadays, the world population is growing older rapidly as people live longer due to better healthcare. Aging leads to progressive

changes in heart structure and deterioration of heart function**.** As a result, aging is the dominant risk factor for the heart disease. The incidence and prevalence of heart disease increase with age, and heart disease is one of the leading causes of death worldwide (Yazdanyar and Newman 2009).

Proper management of the aging heart and prompt treatment of heart disease are vital to extend the healthy lifespan of older adults and patients with heart disease, respectively. Unfortunately, there is no definitive aging heart therapy. On the same note, pharmacological and surgical interventions can achieve limited results in mending the ailing heart as these therapies primarily help to control the signs and symptoms, but fail to reverse the pathological changes and structural damage. The long waiting list is hindering the patients with advanced heart disease from getting a heart transplant.

Despite a significant reduction in cardiovascular mortality over the last decades, especially in the developed countries, cardiovascular disease remains the leading cause of death in many parts of the world (Mc Namara et al. 2019). Thus, stem cell therapists have focused on the developing novel cell-based treatment strategy for a wide range of heart diseases. Mesenchymal stem cells (MSCs) and mononuclear cells isolated from the bone marrow and cord blood are more commonly used to treat heart disease. In fewer studies, cardiac stem cells (CSCs), embryonic stem cell (ESC)-derived cardiac progenitor cells, and skeletal myoblasts also have been trialed to treat heart disease.

Stem cells modulate cardiac regeneration primarily through paracrine signaling (Gallina et al. 2015). The stem cell secretome consists of two major components, i.e., soluble proteins and extracellular vesicles (Maacha et al. 2020). However, there is inadequate evidence showing that the transplanted cells differentiate into cardiomyocytes to ameliorate the ailing heart, as indicated by the poor survival of transplanted cells (Abdelwahid et al. 2016). Furthermore, the wound environment with intense inflammation, hypoxic, and nutrient deprivation is hostile to the transplanted cells which are not prepared for such condition.

In this book chapter, we have discussed the effects of aging on the heart, the pathophysiology of heart diseases, and the contemporary treatment strategies with emphasis on the clinical evidence of using stem cell therapy to repair the damaged heart.

Aging of the Heart

Aging leads to progressive decline in body physiological function, eventually causing various diseases and health complications. For example, aging significantly affects the health of the heart, causing more inferior cardiac function and contributing to the development of heart failure and atrial fibrillation (AF) (Strait and Lakatta 2012; Steenman et al. 2017). Characteristics of the aging heart include alteration in left ventricular diastolic function, left ventricular hypertrophy, reduction of left ventricular systolic reserve, reduction of myocardial contractility, decrease in

maximum heart rate, and decrease in maximum left ventricular ejection fraction (LVEF) (Steenman and Lande 2017; Christou and Seals 2008; Chiao and Rabinovitch 2015).

Reduction of left ventricular diastolic filling rate is the first physiological modification observed at the early stage of aging. The reduction of left ventricular diastolic filling rate is compensated by increasing the atrial contraction in order to sustain the stroke volume and to maintain the LVEF (Fleg and Strait 2012). An increase in atrial contraction is associated with atrial hypertrophy and dilation. In addition, the adrenergic signaling will change to reduce the maximum heart rate to permit a longer filling time (Strait and Lakatta 2012). However, left ventricular contractility and response to β-adrenergic receptor activation reduced with age (Lakatta and Levy 2003). Hence, the myocardial mass will increase to compensate for the reduction in cardiac output. Ventricular hypertrophy is the result of an increase in the size and number of cardiomyocytes. Ventricular hypertrophy is reversible when caused by physiological changes such as in healthy athletes and pregnant women or irreversible when the modification is induced by pathology such as heart disease (Marketou et al. 2016). Although ventricular hypertrophy may provide temporary improvement in cardiac output, however, it will lead to deterioration of cardiac function, i.e., defective ventricular relaxation and filling and even heart failure, in the long term (Tardiff 2006).

The heart undergoes complex changes at the cellular and molecular level during aging. With age, there will be alteration in cellular composition resulting in a reduction of cardiomyocyte population as more cells undergo apoptosis, necrosis, and a decrease in CSC reservoir (Daniele et al. 2004; Chiong et al. 2011). These are due to the aging cardiomyocytes that are more susceptible to stress, including oxidative stress. Therefore, an increase in reactive oxygen species (ROS) production due to aging often results in stimulation of cardiomyocyte death. During cardiomyocyte necrosis, various toxic cellular components that can affect the survival of neighboring cardiomyocytes are released (North and Sinclair 2012). The remaining cardiomyocytes will become hypertrophic to compensate for the reduction in cell number. In addition, there will be changes in the collagen composition with a shift toward type I collagen and increased tissue fibrosis. Fibrotic tissue is linked with poorer myocardial contractility. The accumulation of amyloid protein in the aging myocardium is also related to reducing myocardial contractility (Steenman and Lande 2017).

Cardiac aging is also associated with mitochondrial dysfunction. Tocchi et al. have mentioned that aging mitochondria have inferior functionality, higher production of ROS, higher mitochondrial DNA mutation, higher respiratory chain dysfunction, dysregulation of mitochondrial fission and fusion, as well as suppressed mitophagy (Tocchi et al. 2015). All these lead to the accumulation of dysfunctional mitochondria. Furthermore, dysregulation in calcium signaling, neurohormonal signaling, and nutrient and growth signaling are also related to the impaired cardiac function (Steenman and Lande 2017; Chiao and Rabinovitch 2015). Figure 1 shows the changes in the aging heart at the cellular and tissue level.

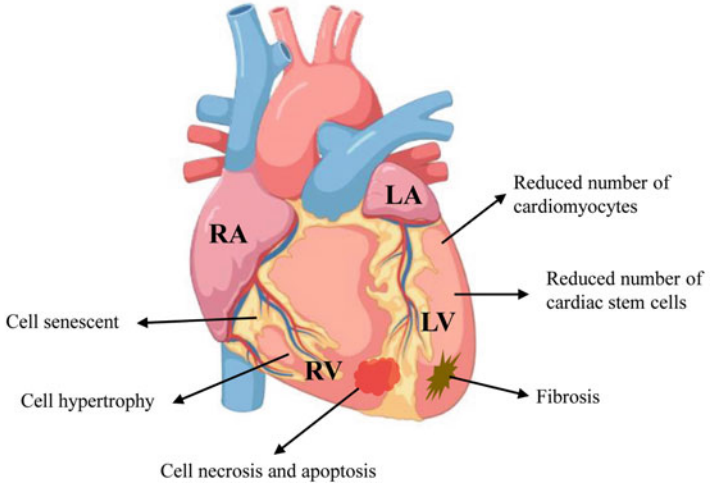

Fig. 1 Changes in aging heart at the cellular and tissue level. With age, the cardiomyocytes will undergo senescence, and the number of cardiomyocytes will reduce as the number of apoptotic and necrotic cells increases. The heart tissue also will become hypertrophic, ischemic, and fibrotic. These pathophysiological changes to the heart tissue are closely related to the changes in aging vascular system whereby the lumen is narrowed by the atherosclerotic plague, leading to poorer perfusion to the heart tissue

Heart Disease

Heart disease is prevalent and is one of the leading causes of death worldwide. There are many types of heart disease, e.g., myocardial infarction (MI) and heart failure, which affect a different part of the organ. MI, commonly known as heart attack, is caused by a sudden disruption in blood supply to the myocardium. The lack of oxygen leads to irreversible damage to the cardiomyocytes. At the cellular and tissue levels, prolonged ischemia causes cardiomyocyte apoptosis and necrosis, inflammation, loss of cellular glycogen, mitochondrial dysfunction, myofibril relaxation, and sarcolemmal disruption. Most of the affected cardiomyocyte cell death took place within the first 24 h of injury. Tissue inflammation following the injury will lead to the second wave of cardiomyocyte cell death. The human heart has inadequate regenerative capacity. Thus, the infarcted tissue will be replaced with fibrous scar tissue. Replacement of functional myocardium with nonfunctional fibrotic scar alters the cardiac contraction and impulse conduction and subsequently increases the prevalence of diastolic and systolic dysfunction, arrhythmia, and heart failure (Thygesen et al. 2018; Frangogiannis 2015).

Heart failure is a clinical syndrome characterized by the inability of the heart to pump sufficient blood to meet the body's metabolic demand. Many factors, including MI, can cause heart failure. Other etiologies include cardiomyopathies, valvular disease, hypertension, diabetes mellitus, myocarditis, infections, systemic toxins,

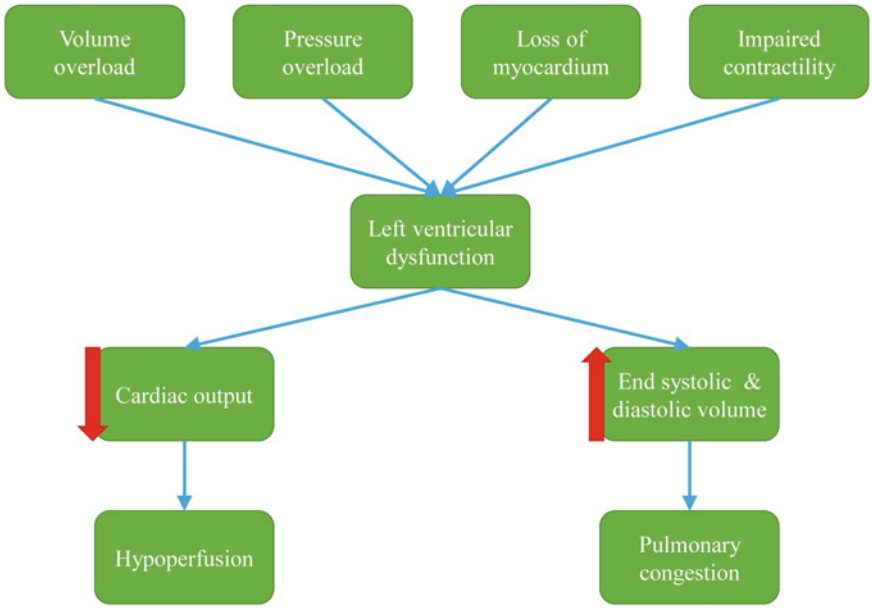

Fig. 2 Pathophysiology of aged and diseased heart. Aging and heart disease will lead to volume overload, pressure overload, loss of myocardium, and reduced contractility which in turn cause left ventricular dysfunction. Left ventricular dysfunction will reduce cardiac output and increase end-systolic and end-diastolic volume which eventually resulted in hypoperfusion and pulmonary congestion, respectively

and cardiotoxic drugs. Generally, the initial insult induced a destructive cycle that causes metabolic and morphological changes in the remaining cardiomyocytes. These cellular changes progressively lead to structural remodeling of the ventricle. Although remodeling initially occurs as a compensation mechanism or adaptive response to sustain cardiac performance, however, over time, the response becomes counterproductive and leads to ultrastructure abnormality, e.g., including hypertrophy and fibrosis, finally causing heart failure. In addition, with time, diastolic dysfunction and subsequent systolic dysfunction resulted in an enlarged, dilated, and low contracting ventricle (Kemp and Conte 2012; Johnson 2014). Figure 2 shows the pathophysiology of an aged and diseased heart.

Conventional Therapies for Heart Disease

Nowadays, treatment for heart disease depends on the type, etiology, symptoms, and severity of the disease. The treatment of ischemic heart disease starts from lifestyle modifications such as regular exercise, smoking cessation, and lipid control. Antiplatelet and antianginal medications are routinely used in those with established diagnosis via coronary angiogram or CT imaging of the heart. Ultimately such a

domain might require percutaneous coronary intervention (stenting) or coronary artery bypass surgery to tackle the blocked native coronary vessels (Dababneh and Goldstein 2020).

Structural heart disease and valvular heart disease deliberately warranted surgical intervention. The progressive valve defect can lead to ventricular dilatation and subsequently dilated cardiomyopathy because of the lengthening and destruction of muscle fibers (Frank-Starling law) (Epstein and Davis 2003). Therefore, cardiothoracic surgeon referral is paramount to address the underlying valve pathology, and valve repair or replacement is mandatory to delineate the structural defect.

Another subgroup of patients commonly seen is those with heart failure due to varying pathology. These patients require medications, e.g., angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), beta-blockers, diuretics, aldosterone antagonists, vasodilators, digoxin, I(f) inhibitor, and angiotensin receptor-neprilysin inhibitor, to control the disease progression and achieve symptom control (Shah et al. 2017). Surgery might have limited benefits for these patients. However, advancements in technologies, such as left ventricular assist devices (LVAD), provide certain advantages in controlling heart failure (Hunt and Ross 2002; Birati and Jessup 2015). Eventually, a heart transplant is the only chance for them. However, there is a critical shortage of donor's hearts worldwide to meet the current demand for heart transplantation.

Mechanisms of Action of Stem Cells in Restoring the Heart Function

Stem cells are multipotent cells that reside in embryos and adult tissues. Stem cells are categorized either by their potency (e.g., totipotent, pluripotent, multipotent, or unipotent) or their source of origin (e.g., embryo, bone marrow, adipose tissue, Wharton's jelly, etc.) (Los et al. 2018). The most widely studied stem cells are MSCs that could be isolated from many adult tissues, e.g., bone marrow, adipose tissue, peripheral blood, skin, and heart. Researchers considered MSCs as the most attractive stem cells because it is easily obtainable from many adult tissues and can be expanded with ease in the laboratory to fetch many cells (Ding et al. 2011; Haider 2018). According to the characterization guideline proposed by the International Society for Cell & Gene Therapy (ISCT), MSCs are cells that (i) are plastic adherent in vitro; (ii) are more than 95% positive of CD105, CD73, and CD90, while less than 2% positive for surface antigen markers CD34, CD45, CD79/CD19, CD14/CD11b, and HLA-DR; and (iii) have in vitro tri-lineage, i.e., chondrogenic, osteogenic, and adipogenic differentiation capability (Horwitz et al. 2005).

Stem cells have been reported to restore heart function via several mechanisms. Generally, the transplanted cells will stimulate proliferation of native cells in the heart and secrete paracrine factors which favor heart repair and regeneration (Fig. 3). Additionally, MSCs can differentiate into cardiomyocytes to repopulate the injured heart tissue (Hafez et al. 2016). However, based on the results from in vivo study, researchers have found that the benefits of MSCs-based therapy do not rely on its

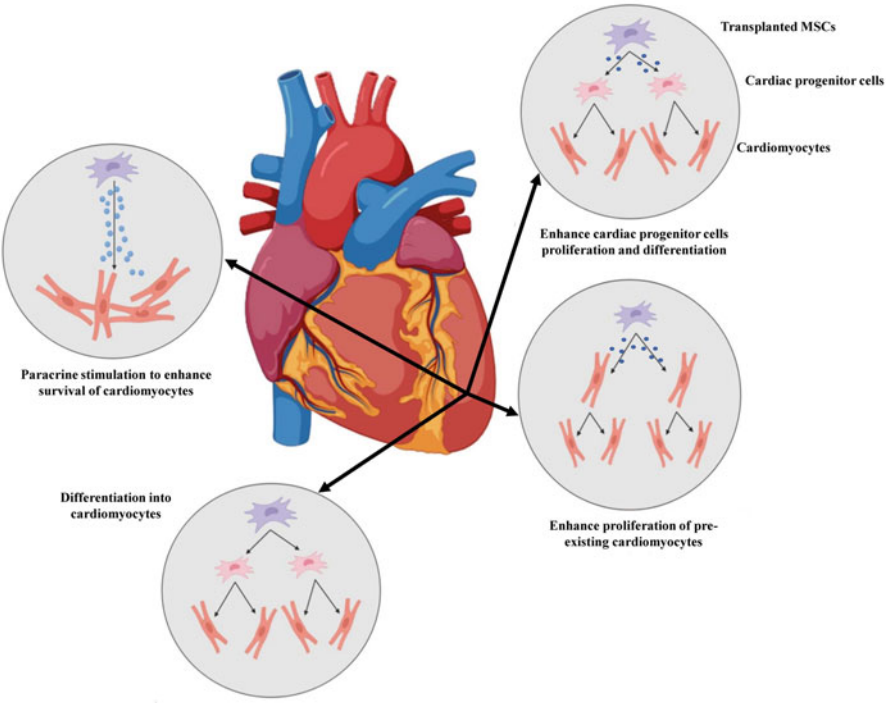

Fig. 3 Functions of stem cell therapy. Transplanted cells secrete paracrine factors and extracellular vesicles that stimulate the proliferation of cardiac progenitor cells and cardiomyocytes and modulate the tissue environment to enhance the survival and function of preexisting cardiomyocytes

ability to repopulate the infarcted heart but more on its secretion of paracrine factors, which possess the immunomodulatory ability and support tissue regeneration. This is evidenced by the poor MSC retention in the infarcted area (Luger et al. 2017).

Despite the poor cell homing in the infarcted area, delivery of MSCs resulted in improved left ventricular function. This improvement has been attributed to the anti-inflammatory effect of MSCs, whereby delivery of MSCs reduces the number of NK cells and neutrophils after MI, subsequently improving left ventricular function (Luger et al. 2017).

The body reacts to injury by producing pro-inflammatory factors such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-17 (IL-17), and interferon-gamma (IFN-γ) (Chaplin 2010). These factors are produced by both innate and adaptive immune cells and attract the migration of MSCs to the injury site. Thus, regardless of the origin, whether exogenously introduced or endogenously recruited, MSCs will be activated by these pro-inflammatory cytokines and polarized to the immunosuppressive phenotype. Upon activation, MSCs will modulate the innate and adaptive immune response by influencing the function of macrophages, natural killer cells, B cells, T cells, mast cells, neutrophils, and dendritic cells (Liau et al. 2020b; Wang et al. 2014).

MSCs mediate immunomodulation through paracrine secretion and cell-to-cell contact (Liau et al. 2020a).

MSCs have been reported to secrete a myriad of anti-inflammatory factors, including galectin-1, interleukin-10 (IL-10), interleukin-13 (IL-13), prostaglandin E2 (PGE2), nitric oxide (NO), transforming growth factor-beta (TGF-β), and indoleamine-2,3-dioxygenase (IDO) (Lim et al. 2018; Kyurkchiev 2014; Haider and Aziz 2017). Even though inflammation is indispensable in wound healing, an overwhelming inflammation upon heart injury is detrimental for the remaining cardiomyocytes. Thus, stem cell therapy, particularly MSCs, can act as a potent modulator of inflammation response to promote heart regeneration. Immunomodulatory role of MSCs is apparent in restoring heart function during heart disease progression.

The paracrine effects of MSCs are not limited to the immune-related cells but including the resident cells, i.e., injured cardiomyocytes and cardiac progenitor cells (CPCs). Exosomes secreted by MSCs contain miR-221 and miR-19a that are involved in apoptosis suppression and activation of PI3K-Akt signaling pathway to promote cardiomyocyte survival and growth (Ward et al. 2018; Yu et al. 2015). The paracrine effects of MSCs are studied via conditioned media exposure in vitro or injection in vivo. Conditioned media are spent media or used media collected from cultured cells. In vitro study showed increased migration and proliferation of CPCs when cultured with MSCs-derived conditioned media. MSCs-derived conditioned media also exert protective effect against serum starvation and hypoxia-induced apoptosis (Nakanishi et al. 2008). On the other hand, in vivo study found that MSCs-derived conditioned media injected in a porcine MI model significantly reduced tissue infarct size and improved systolic function (Timmers et al. 2011). These were achieved by abrogation of TGF-β signaling and apoptosis resulting from phospho-SMAD2 suppression and activation of caspase 3 by the conditioned media (Timmers et al. 2008).

MSCs also improve the myocardial angiogenesis after injury. In a preclinical study using experimental rat model of MI, transplanted MSCs differentiated into endothelial cells and improved the myocardium angiogenesis (Siamak et al. 2003). Nonetheless, most of the studies found that MSCs promote angiogenesis mainly through paracrine signaling (Teng et al. 2015; Cai et al. 2009). MSCs secrete several proangiogenic factors, such as IL-6, TGF-β, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietin-1 (Ang-1), placental growth factor (PGF), hepatocyte growth factor (HGF), and monocyte chemotactic protein-1 (MCP-1) (Maacha et al. 2020; Tao et al. 2016). Moreover, MSCs-derived exosomes are also rich in miRNAs, e.g., miR-210, miR-199-5p, miR-423-5p, miR-939, and miR-21-3p, that are proangiogenic (Baruah and Wary 2020).

Clinical Evidence of Cell-Based Intervention in Ameliorating Heart Disease

Several clinical trials have published their findings, while many more trials are underway and have been registered in the ClinicalTrials.gov database (Table 1) (Rajab et al. 2019). Worth mentioning is the BAMI trial that will recruit 3000 MI

Table 1 List of clinical trials using stem cells to treat cardiac diseases listed in ClinicalTrials.gov database

ClinicalTrials.gov identifier	Phase	Disease	Patients number	Cell source	Cell delivery
NCT00869024	I/II	End-stage HF patients undergoing LVAD implantation	25	Autologous BM-MNCs	10 I/M injections (20×10^6 cells)
NCT01759212	II/III	End-stage HF patients undergoing LVAD implantation	5	Allogeneic BM-MNCs	I/M transplantation
NCT01758406	II	Ischemic HF	50	Autologous CSCs	I/C infusion
NCT02387723	I	Ischemic HF	10	Allogeneic AT-MSCs	I/M transplantation (100×10^6 cells)
NCT01883076	I	HLHS	30	Autologous UC-blood MNCs	I/M transplantation (3×10^6 cells per kg)
NCT01829750	II	HLHS	34	Autologous CPCs	I/C infusion (0.3×10^6 cells per kg)
NCT03406884	I	HLHS	32	Autologous c-kit+ cells derived from the right atrial tissue	6–10 I/M injections (up to 12,500 cells/kg)
NCT03431480	I	HLHS	12	Autologous placenta cord blood MNCs	I/C infusion
NCT03092284	II	HF	81	Allogeneic AT-stem cells	I/M transplantation
NCT03145402	I/II	HF	5	Autologous BM-MNCs	I/C infusion (cells collected from 100–150 ml of BM)
NCT03227198	I/II	HF	5	Autologous BM-MNCs	I/M transplantation
NCT03180450	I/II	HF	60	Allogeneic UC-derived MSCs	Intravenous infusion
NCT02057900	I	HF	10	ESCs-derived CD15+ IsI-1+ progenitors	Fibrin patch transplanted at the epicardium
NCT03763136	-	HF	5	Allogeneic iPSCs-derived CMs	I/M transplantation (100×10^6 cells)
NCT01693042	II/III	Chronic post-MI HF	81	Autologous BM-MNCs	1 or 2 I/C infusions
NCT00644410	1/II	Congestive HF	59	Autologous BMSCs	I/M transplantation
NCT00526253	II/III	Congestive HF post-MI	170	Autologous SkMs	I/M transplantation
NCT02032004	III	Chronic HF due to systolic dysfunction	566	Allogeneic BM-MPCs	T/E transplantation

(continued)

Table 1 (continued)

ClinicalTrials.gov identifier	Phase	Disease	Patients number	Cell source	Cell delivery
NCT02467387	II	Nonischemic HF	23	Allogeneic BMSCs	I/V infusion (1.5×10^6 cells /kg)
NCT03043742	I	Chronic ischemic heart disease patients with laser revascularization surgery	10	Autologous BM-CD133+ cells	Injection into laser channels during trans-myocardial revascularization
NCT04236479	I	Congenital heart disease	36	Allogeneic BMSCs	Cardiopulmonary bypass (1×10^6, 10×10^6, 20×10^6, 40×10^6, 80×10^6 cells/kg)
NCT02501811	II	Ischemic cardiomyopathy	144	Autologous BMSCs or c-kit+ cells or both	15 T/E injections (150×10^6 MSCs, 5×10^6 c-kit+ cells)
NCT00313339	I	MI	31	Autologous BM-CD34+ cells	I/C infusion
NCT00384982	II	MI	116	BM-MNCs	I/C infusion or I/C infusion+ I/M transplantation
NCT02672267	III	MI	50	Allogeneic ischemia-tolerant BMSCs	I/V infusion
NCT01291329	II	MI	160	Allogeneic Wharton's jelly-MSCs	I/C infusion
NCT01392105	II/III	MI	80	Autologous BMSCs	I/C infusion (1×10^6 cells/kg)
NCT02439398	I/II	MI	55	Allogeneic CSCs	I/C infusion (35×10^6 cells)
NCT00313339	I	MI	31	Autologous BM-CD34+ cells	I/C infusion
NCT01187654	II/III	MI	80	BM-AC 133+ or MNCs	I/C infusion
NCT01569178	III	MI	350	Autologous BM-MNCs	I/C infusion
NCT00936819	II	MI	47	Autologous EPCs or EPCs overexpressing eNOS	I/C infusion (20×10^6 cells)

Abbreviations: BM, bone marrow; BMSCs, bone marrow-derived mesenchymal stem cells; EPCs, endothelial progenitor cells; HLHS, hypoplastic left heart syndrome; I/C, intracoronary; I/V, intravenous; LVAD, left ventricular assist device; MI, myocardial infarction; MNCs, mononuclear cells; MSCs, mesenchymal stem cells; eNOS, human endothelial nitric oxide synthase; T/E, transendocardial

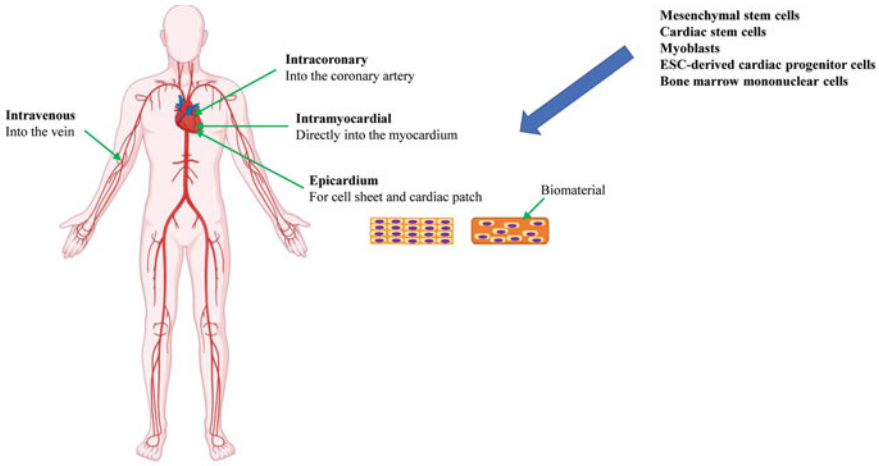

Fig. 4 Stem cell therapy for heart diseases. Mesenchymal stem cells, cardiac stem cells, skeletal myoblasts, embryonic stem cell-derived cardiac progenitor cells, and bone marrow mononuclear cells have been used to treat heart disease. The cells can be administered directly through the intravenous, intracoronary, and intramyocardial routes or assembled as cell sheet and cardiac patch to be transplanted at the epicardium

patients (Mathur et al. 2017). The patients will be divided equally into the stem cell treatment group, which would receive an intracoronary infusion of autologous bone marrow mononuclear cells (BMMCs), and a control group patient who would be given only the standard therapy. The trial aims to determine if BMMC treatment can reduce all-cause mortality in acute MI (AMI) patients with an LVEF of $\leq$45% after successful reperfusion. Another interesting study is the ENACT-AMI trial that applied autologous endothelial progenitor cells overexpressing human endothelial nitric oxide synthase (eNOS) to treat MI patients (Taljaard et al. 2010). This is the first trial combining cell and gene therapy to treat heart disease.

Multiple types of stem cells, e.g., MSCs and mononuclear cells isolated from different tissue sources, cardiac stem cells, skeletal myoblasts, and ESCs-derived CPCs, have been tested clinically to treat heart disease (Haider 2006). The cells were administered via different route, most commonly via direct transplantation to the myocardium or through the intracoronary infusion. Furthermore, the cells also can be injected intravenously and transplanted as a cell sheet or cardiac patch (Fig. 4) (Guo et al. 2020).

Acute Myocardial Infraction

Bone marrow cells in different forms have been tested in multiple randomized clinical trials to treat AMI. Most of the studies used the heterogeneous cell population, i.e., BMMCs, while some applied specific cell population such as bone marrow-derived mesenchymal stem cells (BMSCs), CD133+ bone marrow progenitor cells, and $CD34^{+}$ $CXCR4^{+}$ bone marrow cells.

Strauer et al. reported the intracoronary infusion of autologous BMMCs in ten AMI patients. They found that cell therapy is safe and effective in reducing the size of the infarcted region and improving cardiac function (Strauer et al. 2002). In another study that also administered autologous BMMCs via intracoronary route, the authors found no changes in left ventricular end-diastolic volume (LVEDV), significant improvement in left ventricular end-systolic volume (LVESV) and LVEF, and better regional contractility in the patients who received cell therapy compared to the patients who received standard therapy (Francisco et al. 2004).

Lipiec et al. infused autologous BMMCs via intracoronary route to 26 AMI patients and found no significant changes in LVEF, LVEDV, LVESV, and left ventricular-wall motion score index (WMSI) compared to the 13 patients in the control group after 6 months (Lipiec et al. 2009). However, the infarct area WMSI, perfusion defect extent, left ventricular perfusion score index (PSI), and infarct area PSI improved significantly in the stem cell group.

The FINCELL trial divided 80 AMI patients equally to the treatment group that received intracoronary BMMC injection and the placebo group that received media without cells (Huikuri et al. 2008). After 6 months, the treatment group showed more considerable improvement in LVEF compared to the placebo group. Ge et al. infused autologous BMMCs into the infarct-related coronary artery of ten AMI patients and found that LVEF increased while left ventricular end-diastolic internal diameter (LVDd) remained unchanged and myocardial perfusion defect scores decreased. This was compared with ten AMI patients who received bone marrow supernatant and showed no changes in LVEF and myocardial perfusion defect scores as well as larger LVDd (Ge et al. 2006). Cao et al. delivered autologous BMMCs via intracoronary route to 41 AMI patients as compared to normal saline solution without cells to 45 AMI patients in the control group (Cao et al. 2009). After 6 months, the LVEF improved significantly in the stem cell treatment group compared to the control treatment group. The improvement persisted for up to 4 years of follow-up.

The HEBE trial recruited 200 AMI patients and randomly assigned them to receive either an intracoronary infusion of BMMCs, peripheral blood mononuclear cells, or standard therapy (Hirsch et al. 2010). The researchers observed no significant difference in the percentage of dysfunctional left ventricular segments that improved after treatment. Similarly, there was no observed difference between the cell treatment groups in terms of improvement in LVEF and changes in left ventricle mass, volume, and infarct size at 4 months after treatment as compared with the control group. The ASTANI trial treated 50 AMI patients with an intracoronary infusion of autologous BMMCs and found that stem cell therapy significantly increases the exercise time, peak heart rate, and percentage of heart rate reserves compared to the control group (50 patients) 6 months after the treatment (Lunde et al. 2007). However, no significant differences were detected for the changes in LVEF, LVEDV, WMSI, and infarct size between the two groups at 6 and 12 months (Lunde et al. 2006, Lunde et al. 2008). The REGENT trial involved intracoronary infusion of BMMCs and CD34+ CXCR4+ bone marrow cells to 80 AMI patients each (Tendera et al. 2009). At 6 months, the LVEF increased by 3% in both stem cell therapy

groups but remained unchanged in the 40 control group patients. However, the observed improvement was statistically insignificant. Also, no significant changes were observed in LVESV and LVEDV. Nonetheless, the authors reported that stem cell therapy gave better results in patients with extremely poor LVEF.

In a study examining the effect of BMMC dosage, 66 MI patients were divided equally into 3 treatment groups, high-dose group (100×10^6 cells), low-dose group (10×10^6), and a control group (without cell transplantation) (Meluzín et al.2006). All the three groups had intravenous administration of their respective treatment. The results showed that the high-dose group demonstrated significantly higher improvement in the peak systolic velocity of longitudinal contraction of the infarction wall compared to the low-dose group, and only the high-dose group showed considerable enhancement in LVEF compared to the control group after 3 months of follow-up. The significant improvement in LVEF persisted for up to 12 months (Meluzín et al. 2008). In a study that compared the effectiveness of intracoronary and intravenous administration of BMMCs, the authors reported no significant differences in the changes in echocardiographic parameters, i.e., LVEF, LVEDV, LVESV, and WMSI, at 6 months between the intravenous group, intracoronary group, and control group (Nogueira et al. 2009).

Many have raised the question regarding the optimal timing for stem cell therapy in post-MI patients. Thus, the TIME trial was designed to examine the safety and efficacy of intracoronary infusion of 150×10^6 autologous BMMCs at day 3 or 7 post-MI on 120 patients (Traverse et al. 2012). After 6 months, it was found that the timing of cell infusion does not affect the improvement in global and regional left ventricular function, whereby no significant differences were observed between the stem cell-based treatment groups. Furthermore, no significant differences were detected between the stem cell groups and the placebo group at 6 months and 2 years (Traverse et al. 2012, Traverse et al. 2018). The LateTIME trial used a similar treatment protocol; however, the cells were administered 2–3 weeks post-MI (Traverse et al. 2010). Similarly, results showed no differences in global and regional left ventricular function between the groups after 6 months (Traverse et al. 2011). The SWISS-AMI trial randomized 200 AMI patients in ratio 1:1:1 to receive early (5–7 days after AMI) administration of BMMCs, late (3–4 weeks after AMI) administration of BMMCs, and standard therapy (control group). At 4-month follow-up, there was no real difference in left ventricular function improvement between the two treatment groups and the control group (Sürder et al. 2013). Similarly, no improvement was observed during 12-month follow-up (Sürder et al. 2016). Thus, the timing of cell transplantation might be less important when using bone marrow cells to treat AMI.

The BONAMI trial recruited 101 AMI patients divided into the stem cell therapy group (52 patients) who received an intracoronary infusion of autologous bone marrow cells ($98.3 \pm 8.7 \times 10^6$ cells) and control treatment group (49 patients) who received the sham infusion without cells (Roncalli et al. 2010). The authors reported that cell therapy significantly improved myocardial viability after 3 months. However, no differences were detected for the LVEF, global WMSI, and infarct size. The REGENERATE-AMI trial investigated intracoronary infusion of autologous

bone marrow cells to 55 AMI patients. Another 45 AMI patients in the placebo treatment group received normal saline without cells (Choudry et al. 2016). The results showed that the stem cell therapy group had greater improvement in LVEF and significantly higher myocardial salvage index than the placebo treatment group. In the BOOST trial that treated 30 AMI patients with an intracoronary infusion of autologous bone marrow cells, the researchers found that cell therapy is safe and helped to improve the diastolic function and LVEF compared to the control patients (30 patients) (Schaefer et al. 2006, Wollert et al. 2004). However, the follow-up study found that the significant improvement in LVEF diminished after 18 months (Meyer et al. 2006, Meyer et al. 2009). Nonetheless, the improvement in LVEF was maintained in the subgroup of patients with more transmural infarcts. In the multiple-arm BOOST-2 trials, the researchers found that intracoronary infusion of high dose and low dose of autologous bone marrow cells, γ-irradiated or not, did not significantly improve the LVEF (Wollert et al. 2017).

The LEUVINE-AMI trial applied autologous bone marrow-derived stem cells in 33 AMI patients, and another 34 patients in the control group received the placebo (Janssens et al. 2006). Stem cell-based treatment significantly reduced the myocardial infarct size and improved the regional systolic function compared to the placebo treatment group after 4 months. However, no significant differences were detected in LVEF, LVEDV, and LVESV. Grajek et al. intravenously infused bone marrow stem cells to 31 AMI patients. The authors found that it did not result in any significant improvement in LVEF, LVEDV, and LVESV compared to the 14 patients in the control group during 1-year follow-up (Grajek et al. 2009).

In the multicenter REPAIR-AMI trial, 101 patients received bone marrow-derived progenitor cell infusion into the infarct-related coronary artery, and another 103 patients received placebo treatment (Schächinger et al. 2006). The progenitor cell-based therapy significantly improved LVEF, LVESV, and regional contractility compared to the placebo treatment at 4 months. Furthermore, at 2 years, the progenitor cell-based therapy group demonstrated a lower incidence of recurring MI, rehospitalization for heart failure, and death as well as higher improvement in LVEF compared to the placebo-treated group (Assmus et al. 2010). In the TOPCARE-AMI trial, 59 patients with AMI received an intracoronary infusion of circulating progenitor cells or bone marrow-derived progenitor cells and are followed of up to 5 years (Leistner et al. 2011; Schächinger et al. 2004). The results showed the long-term safety of intracoronary delivery of autologous circulating progenitor cells and bone marrow-derived progenitor cells in AMI patients. In addition, the patients also showed a sustained improvement in LVEF for 5 years without any significant difference between the two treatment groups.

Chen et al. examined the potential of using autologous BMSCs to treat AMI by intracoronary administrating the cells to 34 AMI patients with another 35 AMI patients in the control group receiving only the saline (Chen et al. 2004). Patients who received the stem cell-based therapy demonstrated significant improvement in LVEF, wall movement velocity over the infarcted region, LVEDV, and LVESV after 3 months compared to those in the control group.

Bartunek et al. reported that intracoronary infusion of CD133+ bone marrow progenitor cells in 19 AMI patients significantly improved the LVEF, left ventricular regional chordae shortening, and reduction of the perfusion defect compared to the 16 AMI patients without the stem cell therapy (Bartunek et al. 2005). However, it also increased the incidence of coronary events. In another study, the researchers compared intracoronary infusion of CD133+ cells from bone marrow and peripheral blood with the standard therapy (five patients each group) (Colombo et al. 2011). It was found that LVEF and wall motion score index remained stable for all groups, but the infarct-related myocardial blood flow was only increased in the bone morrow group after 1 year. In addition, infarct size and summed rest score decreased most significantly in the bone marrow-treated group.

Overall, some studies such as BOOST, TOCARE-AMI, FINCELL, REGENERATE-AMI, and REPAIR-AMI demonstrated improvement in global and regional left ventricular function after the bone marrow cell therapy. Nonetheless, there are also many trials, i.e., BONAMI, LEUVEN-AMI, HEBE, ASTAMI, BOOST-2, TIME, LateTIME, SWISS-AMI, and REGENT, which failed to detect significant functional improvement. Furthermore, in the systematic review and meta-analysis conducted by Fisher et al. in 2015, the authors recovered 41 randomized controlled trials (RCT) that compared the safety and efficacy of autologous bone marrow cells with no cell therapy group and found that there is insufficient evidence to prove the advantages of applying autologous bone marrow cells in MI patients (Fisher et al. 2015). These discrepancies might be due to the differences in the cell preparation technique, the number of cells administered, cell composition, and timing of cell administration.

Heart Failure

The FOCUS-CCTRN trial studied the safety of transendocardial injection of autologous BMMCs in end-stage ischemic heart disease patients (Perin et al. 2012). Results indicated that the mononuclear cells were well-tolerated, and the treatment showed a positive effect on myocardial perfusion and contractility. The investigators reported that the cell therapy resulted in a slight improvement in patients' LVEF but did not reduce LVESV or increase maximal oxygen consumption. In addition, the clinical trial also showed a positive correlation between the degree of LVEF improvement and the percentage of $CD34^+$ and $CD133^+$ cells. Specifically, every 3% increase in CD34 or CD133 cells was associated with an increase in LVEF of 3–5.9%. These critical findings suggested that the cellular composition does affect the clinical effectiveness of stem cell therapy. In 2016, Fisher et al. published a systematic review and meta-analysis that included 38 randomized controlled trials (RCTs) and 1907 participants. The authors concluded that BMSCs therapy could reduce mortality and improve the cardiac function of patients with chronic ischemic heart disease and heart failure, albeit with the low quality of the collected evidence (Fisher et al. 2016). The BMSCs used in these RCTs included BMSCs, BMMCs, $CD133^+$ cells, $CD34^+$ cells, aldehyde dehydrogenase-bright cells, bone marrow aspirate

concentrate, and G-CSF mobilized and cultured circulating mononuclear pro-angiogenic cells.

Interestingly, bone marrow stem cells have been cardiomyogenically induced for the treatment of heart failure clinically. The C-CURE trial used cardiopoietic stem cells (cardiomyogenic differentiated BMSCs) to treat patients with chronic heart failure (Bartunek et al. 2013). The 2-year follow-up showed that cell therapy is safe. In addition, the efficacy measurement at 6 months showed that LVEF increased, LVESV decreased, and 6-min walk distance improved significantly compared to the control group. Instead of expanding the cells in normoxic conditions like most studies, Butler et al. cultured the allogeneic BMSCs in hypoxic condition and used the cell to treat nonischemic cardiomyopathy (Javed et al. 2017). Treatment with ischemia-tolerant BMSCs is safe and significantly improved the 6-minute walking distance and Kansas City Cardiomyopathy Questionnaire clinical summary score compared to the control group.

Apart from bone marrow stem cells, umbilical cord-derived mesenchymal stem cells (UC-MSCs) also have been used to treat heart failure. The RIMECARD trial applied allogeneic UC-MSCs to treat 15 patients with heart failure (Bartolucci et al. 2017). The cell therapy group demonstrated significant improvement in LVEF, New York Heart Association (NYHA) functional class, and Minnesota Living with Heart Failure Questionnaire (MLHFQ) score. In a separate study, Zhao et al. administered UC-MSCs to treat chronic systolic heart failure. The authors reported improved 6-minute walk distance and LVEF improved besides significantly reduced mortality rate compared to the control group (Zhao et al. 2015).

In 2015, Menasché et al. reported the first clinical case of using human embryonic stem cell (ESC)-derived CPCs to treat severe heart failure (Menasche et al. 2015). The patient showed improvement in NYHA functional class and LVEF. More importantly, no adverse event was reported.

Refractory Angina

Refractory angina (RA) is a chronic condition ($\geq$3 months) characterized by angina in the setting of coronary artery disease (CAD). Currently, RA cannot be controlled by conventional therapeutic interventions, angioplasty, coronary artery bypass surgery, or a combination of the two methods mentioned above. Hence, Losordo et al. conducted a phase II, randomized, controlled clinical trial involving 26 centers (167 patients) in the United States to treat patients with chronic RA with autologous stem cells (Losordo et al. 2011). In this study, patients received either low dose (1×10^5 cells/kg) or high dose (5×10^5 cells/kg) of G-CSF mobilized autologous $CD34^+$ stem cells through the intramyocardial route. The 6-month and 12-month follow-up showed that only the low-dose group recorded a significant reduction in angina frequency and improvement in exercise tolerance. Similarly, the RENEW trial and clinical trial by Wang et al. that also administered autologous $CD34^+$ stem cells showed a reduction in angina frequency, improvement in exercise tolerance, and improvement in the Canadian Cardiovascular Society (CCS) class compared to the placebo group (Povsic et al. 2016, Wang et al. 2010).

Mathiasen et al. found benefit in treating patients with CAD and RA using BMSCs (Mathiasen et al. 2013; Haack-Sorensen et al. 2013). The results from the 3-year observation period showed that the cell therapy reduced the frequency of hospital admission, improved the exercise time, lowered the CCS class, increased the Seattle Angina Questionnaire (SAQ) scores, and exhibited long-term safety with no adverse effect. Other investigators assessing the use of bone marrow-derived stem cells to treat RA also reported encouraging results (Beeres et al. 2006; Vicario et al. 2004; Tse et al. 2007; van Ramshorst et al. 2009; Pokushalov et al. 2010).

The PROGENITOR trial and REGENT-VSEL trial applied transendocardial administration of autologous bone marrow $CD133^{+}$ cells to treat patients with RA (Wojciech et al. 2017, Pilar et al. 2014). The results showed that cell therapy reduced frequency of angina and improved the CCS class in the PROGENITOR trial. Still, fewer promising findings were reported in the REGENT-VSEL trial that found no significant difference in single-photon emission computed tomography (SPECT) score, left ventricular function, and CCS class compared to the control group.

In a recent clinical trial (MyStromalCell trial), Qayyum et al. reported the intramyocardial administration of autologous adipose-derived stromal cells (ASCs) to treat 40 patients with RA with another 20 patients in the control group receiving normal saline (Qayyum et al. 2019). Patients treated with ASCs demonstrated improved metabolic equivalents, CCS class, and NYHA class compared to the control group during the 3-year observation period. In addition, the ASCs-treated group was able to maintain the exercise capacity, while deterioration was recorded in the control group. In the ATHENA trial that also used ASCs to treat RA, the authors found that more patients demonstrated improvement in HYHA class and CSS class than the placebo group (Henry et al. 2017).

Limitations of Stem Cell Therapy

Despite significant progress in clinical translation of cell therapy in heart disease over the past decade, many uncertainties remain regarding the most efficacious cell type, cell dosage, and route and timing of cell administration (Haider and Ashraf 2005). Adding to the complexity, there is growing evidence showing that stem cells harvested from elderly patients do not produce the same benefits from healthy donors (Haider et al. 2018). Collectively, these issues highlighted the need to investigate further the mechanisms underlying stem cell survival, plasticity, and functionality.

The most critical question yet to be answered is to find an ideal type of stem cells to treat heart diseases. For this purpose, it is crucial to understand the mechanisms of each type of stem cells in affecting the myocardial performance and also in modulating different cardiac pathologies. Different types of cells might be needed for diverse cardiac pathologies. Thus far, bone marrow-derived stem cells have been widely used clinically, and they have been proven safe and beneficial under certain circumstances. However, the cells' regeneration potential is controversial. CSCs can

be patient-specific, but the tissue collection procedure is highly invasive, and the culture procedure needs further optimization, especially the upscaling process. An alternative option is to use the cardiomyogenic differentiated MSCs. In fact, the C-CURE trial has used the cardiomyogenic differentiated BMSCs to treat heart failure (Bartunek et al. 2013).

Another key factor for successful cell-based therapy is determining the optimal route of delivery. Cells can be injected intravenously, intracoronary, or directly into the myocardium, each with distinct pros and cons. Cell engraftment at the injury site majorly depends on the delivery strategy. Generally, cell engraftment improved through intramyocardial injection under direct vision at the injury site. Scaffold-based cell delivery strategy is gaining popularity as it promotes the rate of cell survival and integration of transplanted cells in the hostile host environment besides ensuring site-specific delivery (Kc et al. 2019). In addition, the cells also can be expanded and transplanted as a monolayer or multilayer cell sheet (Guo et al. 2020). This method of cell delivery reduces the need for the transplanted cells to migrate long distance to home into the injured myocardium to participate in the repair process (Meluzin et al. 2006; Nogueira et al. 2009). Kanelidis et al. in their meta-analysis of preclinical and clinical studies found that intramyocardial injection of stem cells through catheter-based transendocardial stem cell injection provided more benefits than the intravenous route in terms of infarct size reduction and LVEF improvement (Kanelidis et al. 2017).

A few clinical trials have examined the effects of different cell dosages for cardiac regeneration (Meluzin et al. 2006). Averse to the findings of Meluzín et al. that found that the high-dose treatment is more effective in treating MI, Losordo et al. reported that the low-dose cell therapy gave better results in a patient with RA. The discrepancy could be due to their use in different cardiac pathologies and diverging routes of cell administration. For example, more cells are needed when using the intravenous and intracoronary routes of cell delivery compared to intramyocardial transplantation due to lower cell engraftment and vast distribution in nontargeted organs besides massive cell death post engraftment. Pre-conditioning protocols are being developed and optimized to support donor cell survival (Haider and Ashraf 2012) via various mechanisms including induction of pro-survival microRNAs (Kim et al. 2009).

Lastly, stem cells isolated from different individuals might vary in cellular composition, characteristics, and functionality. These variations contributed to the outcome disparity. Quality of the cell preparation remains an ultimate determinant of the outcome and success of cell-based therapy (Haider 2017). It is well known that human stem cells become less viable and dysfunctional with age and individuals with chronic diseases (Efimenko et al. 2015; Shahid and Haider 2016). Such conditions may alter the effectiveness of stem cell therapy. Therefore, the efficacy of autologous stem cells used in previous studies to treat older patients with heart disease may be inconsistent. Thus, the considerations mentioned earlier should be taken into account while interpreting the data of previous studies and designing future research for heart diseases besides developing strategies to restore reparability characteristics of the aging cells (Igura et al. 2011).

Future Perspectives

Supported by the advancement and introduction of new technology, various pharmacologic and genetic strategies are being developed to improve the currently available cell-based therapy for heart disease. These efforts include combination with gene therapy and biomaterial as well as application of stem cell-derived extracellular vesicles.

Combination with Gene Therapy

Recent advancements in high-precision genome-engineering CRISPR/Cas9 system have allowed the researchers to design gene therapy to up- or downregulate the expression of cardiac, vascular, or immune system-relevant gene(s) which is/are abnormally expressed and permanently correct disease-causing mutations in the adult cardiomyocytes. However, despite the tremendous promise, most studies encountered difficulties translating gene therapy to the clinical setting. For example, the recent CUPID2 trial that applied gene therapy (AAV1/SERCA2a) showed low efficacy in heart failure patients despite having encouraging results during the experimental preclinical studies (Greenberg et al. 2016). Discouraging results were also reported in the STOP-HF trial (Chung et al. 2015). Other gene-based innovations such as lineage reprogramming sound promising in theory. However, such intervention could introduce ectopic cardiomyocyte formation, and the activity of the transferred genes is not well-established.

Thus, a novel combinatorial approach of gene therapy and stem cell treatment may prove to be most feasible and efficacious (Haider et al. 2011). The combination therapy offers the advantage that cells can be genetically engineered ex vivo before transplantation, offering a safer alternative and more precise control of gene expression than gene therapy alone (Jiang et al. 2006; Haider et al. 2008). Another potential strategy involves genetically engineered cell grafting using a mixture of physiologically relevant cell types, including stem cells, cardiomyocytes, and neuronal, vascular, and immune cells (Hosseini et al. 2018). These cells serve as vehicles to deliver the gene(s) or even microRNAs of interest to the heart and provide a more comprehensive regenerative strategy compared to each cell type without genetic modulation (Kim et al. 2012a, b).

Exosomes

Exosomes are membrane-bound vesicles produced by cells and contain a variety of factors, including nucleic acids, lipids, and proteins which are considered to be primarily responsible for intercellular transfer of bioactive molecules. More recently, exosomes-based research has gained intense interest as a fast-emerging cell-free therapy approach for many diseases, including heart disease (Haider and Aslam 2018).

The systematic review and meta-analysis conducted by Zhang et al. found that MSCs-secreted exosomes improved the heart function in experimental animal model of myocardial ischemia-reperfusion injury (Zhang et al. 2016). Similarly, CSC-secreted exosomes were also reported to promote heart regeneration in experimental mouse model of MI (Ibrahim et al. 2014). Adamiak et al. reported that induced pluripotent stem cell (iPSC)-secreted exosomes are more potent than iPSCs in myocardial reparability in vivo (Adamiak et al. 2018). Besides being equal or better than the original cells in cardiac protection, the use of exosomes is also safer by mitigating the risk of potential adverse effects observed in cell therapy, such as host immune rejection and tumor formation (Haider and Aramini 2020). These exciting results await future comprehensive clinical evaluations as most of the current findings are still at the preclinical stage.

Biomaterials

Retention and engraftment of transplanted cells at the injury site are crucial to maintaining the therapeutic effects. Unfortunately, regardless of the route of administration, the majority of the cells do not reach (intravenous and intracoronary injection) or engraft (intramyocardial injection) at the damaged myocardium. The poor cell engraftment limited the therapeutic benefits of stem cell therapy. Therefore, scaffolds, either in the form of nanofibers, cell sheets, biodegradable hydrogels, or decellularized tissues, have been utilized to deliver and promote cell retention in the infarcted myocardium. The scaffolds can be made from various biodegradable biomaterials, such as the natural alginate, collagen, and Matrigel, as well as synthetic polycaprolactone (PCL) and poly-L-lactic acid (PLLA) (Reis et al. 2016). An ideal scaffold should be biodegradable and biocompatible having appropriate thickness and could provide mechanical support, besides being easy to handle and permitting precise placement. Currently, research is focused to develop an ideal scaffold for cardiac tissue engineering.

Conclusion

The contemporary treatment options show limited success for the treatment of heart diseases. Stem cell-based therapy has emerged as a novel approach. Even though the quality and quantity of the available data, especially the clinical data, are still limited, the results reported thus far are promising. Further in-depth mechanistic studies are warranted in the future to understand how stem cells work and to optimize the treatment protocol to improve the safety and efficacy of stem cell-based therapy. A combination of stem cells with gene therapy and biomaterial might be able to create a synergistic effect and is an area that needs to be further explored. Also, more efforts are needed to develop the cell-free exosome therapy to ensure its safety and efficacy before being used clinically as an alternative for stem cell therapy.

Cross-References

- ▶ Augmenting Mesenchymal Stem Cell-Based Therapy of the Infarcted Myocardium with Statins
- ▶ Considerations for Clinical Use of Mesenchymal Stromal Cells
- ▶ Mesenchymal Stem Cells for Cardiac Repair
- ▶ Molecular Signature of Stem Cells Undergoing Cardiomyogenic Differentiation
- ▶ Stem Cell Applications in Cardiac Tissue Regeneration
- ▶ Therapeutic Uses of Stem Cells for Heart Failure: Hype or Hope

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Mesenchymal Stem Cells for Cardiac Repair 10

The Clinical Perspective

Abdullah Murhaf Al-Khani, Mohamed Abdelghafour Khalifa, and Khawaja Husnain Haider

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Abstract

Cardiovascular diseases (CVDs), particularly acute and chronic ischemic heart disease (IHD), are the primary cause of morbidity and mortality worldwide. The contemporary pharmacological and invasive revascularization strategies, i.e., percutaneous coronary angiography (PCA) and coronary artery bypass grafting (CABG), reduce mortality and could only provide symptomatic relief. Cell-based therapy has emerged as a novel breakthrough strategy to ensure angiomyogenic repair of the ischemically damaged heart via the generation of neomyocytes and biological bypassing to restore regional blood flow. In this regard, bone marrow (BM)-derived mesenchymal stem cells (MSCs) have shown promise and progressed to advanced phases of clinical assessment. MSCs are one of the well-

A. M. Al-Khani · M. A. Khalifa
Sulaiman AlRajhi Medical School, Al Bukairiyah, Kingdom of Saudi Arabia

K. H. Haider (✉)
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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studied and characterized cell types in vitro and various small experimental animal models and during the translational animal models for safety and reparability. They are currently the most used cells for cell-based cardiovascular therapy with excellent safety profiles, immunomodulatory properties, paracrine action, and differentiation potential. With overwhelming success, researchers have advanced their efforts to bring MSCs a step closer to their eventual routine use in the clinics. This chapter summarizes the advancement of MSCs from in vitro characterization to the clinical phase and discusses their future perspective.

Keywords

Bone marrow · Cell therapy · Heart failure · Infarction · In vitro · Ischemia · Large animal models · Mesenchymal · MSCs · Small animal models · Stem cells

Abbreviations

6-MWT	Six-minute walk test
AMI	Acute myocardial infarction
Ang-1	Angiopoietin-1
BM	Bone marrow
Brdu	5-Bromodeoxyuridine
CFU-F	Colony-forming units-fibroblastic
CVDs	Cardiovascular diseases
eGFP	Enhanced green fluorescent protein
FGF	Fibroblast growth factor
GLP-1	Glucagon-like peptide-1
Glut	Glucose transporter
HSCs	Hematopoietic stem cells
IGF-1	Insulin-like growth factor-1
IHD	Ischemic heart disease
ISCT	International Society for Cell Therapy
LAD	Left anterior descending
Lin-	Lineage negative
LV	Left ventricle
LVEF	Left ventricular ejection fraction
MDCT	Multi-detector computed tomography
MHC	Major histocompatibility complex
miR	MicroRNA
MSCs	Mesenchymal stem cells
M-SPECT	Myocardial single-photon emission tomography
NPY	Neuropeptide Y
RBCs	Red blood cells
SAE	Serious adverse events
TGF-b	Transforming growth factor-b
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling

VEGF	Vascular endothelial growth factor
WNT	Homologous of wingless and Int-1

Introduction

Cardiovascular diseases (CVDs) are the leading cause of mortality globally (WHO 2017) as they account for nearly 836,546 deaths in the USA alone, which is equivalent to one out of every three deaths (AHA-2018 Statistics at a glance). Among the CVDs, ischemic heart disease (IHD) is a major clinical challenge that causes morbidity and mortality in patients in acute and chronic forms. Thrombotic coronary occlusion due to atherosclerotic rupture leads to acute myocardial infarction (AMI) due to compromised blood supply to the affected region of the heart, thus causing massive cardiomyocyte death (Falk et al. 1995). Although the number of surviving patients, their quality of life post-AMI, and the rate of rehospitalization due to recurrence of AMI episodes improved due to advancements in contemporary revascularization techniques and better pharmacological management, these interventions only provide symptomatic relief to the patients. The infarcted myocardium undergoes a series of detrimental events as part of the intrinsic repair process. This involves forming a noncontractile cicatricle tissue to replace the damaged myocardium besides undergoing geometrical changes in the myocardium to accommodate altered pressure-volume needs and overstretching of viable cardiomyocytes, especially in the peri-infarct region to sustain near-normal contractile function (Taggart 2012; Maron et al. 2006).

Standard heart failure medical therapies suffer from a lack of potential to replenish the dead cardiomyocytes. Hence, the emerging strategy of cell-based therapy for myocardial repair and regeneration is revolutionary and exploits the exclusive reparability and differentiation potential of stem cells into structurally and functionally competent neomyocytes and neovascular structures to restore the lost myocardial function (Kwon et al. 2010; Haider et al. 2010a). Given their optimal characteristics and ease of availability, bone marrow (BM)-derived mesenchymal stem cells (MSCs) indeed provide an attractive source of cells for heart cell therapy. This chapter gives an overview of the progress of BM-derived MSCs from in vitro (Table 1) to preclinical studies in small and large animal models focusing on their safety and efficacy leading to the published randomized placebo-controlled clinical trials in the human subjects.

BM-Derived MSCs

BM comprises two main lineages of stem/progenitor cells, i.e., hematopoietic stem cells (HSCs) and MSCs (Haider and Ashraf 2005). MSCs were first described by Friedenstein et al. in 1968 as the fibroblast-like colony-forming units (Friedenstein et al. 1968) and later substantiated and characterized by many other research groups (Bobis et al. 2006; Deng et al. 2008; Luu et al. 2007; Bhat et al. 2021). Such characterization is of great significance to ensure uniformity of cell characteristic prepared from various labs (Stroncek et al. 2020) and to ensure quality preparation

Table 1 Summary of some of the studies reporting in vitro differentiation of BM-derived MSCs

Author/year	Cell type	Method of differentiation	Main findings
Wakitani et al. (1995)	Rat BM-derived MSCs	5-Azacytidin treatment	5-Azacytidine treatment at 24 h after seeding of twice-passaged MSCs in vitro culture. After an exposure of 24 h, long, multinucleated myotubes developed in some of the dishes on days 7–11 after treatment
Makino et al. (1999)	Murine BM-derived MSCs	5-Azacytidin treatment	Nearly 30% of the cells connected with adjoining cells after 1 week, formed myotube-like structures, began spontaneously beating after 2 weeks, and beat synchronously after 3 weeks. Expressed ANP and BNP, stained positive for anti-myosin, anti-desmin, and anti-actinin. Revealed a cardiomyocyte-like ultrastructure. Also expressed MEF-2A and MEF-2D
Rangappa et al. (2003)	Human MSCs	Human MSCs and human cardiomyocytes were mixed at a 1:1 ratio in smooth muscle 2 media	Differentiated hMSCs from the coculture expressed myosin heavy chain, beta-actin, and cTnT detected by PCR. Immunostaining also showed myosin heavy chain and cTnT. Only beta-actin expression was observed in the hMSCs incubated with conditioned media without serum
Antonitsis et al. (2007)	Human BM-derived MSCs	Second passaged cells were treated with 10 microM 5-azacytidine for 24 h	MSCs treated with 5-azacytidine became stick-like morphology, connecting with adjoining cells forming myotube-like structures, running in a parallel fashion. Immunohistochemically positive for myosin

(continued)

Table 1 (continued)

Author/year	Cell type	Method of differentiation	Main findings
			heavy chain and vimentin. The mRNAs of alpha-cardiac actin, beta-myosin heavy chain, and cTn-T were also expressed
Ramkisoensing et al. (2012)	Human adipose tissue MSCs and amniotic membrane MSCs with or without Cx-43	Ten-day coculture with neonatal rat ventricular cardiomyocytes	Functional cardiomyogenic differentiation, based on action potential recordings, occurred only in control fetal AM hMSCs. Cx45 overexpression in Cx43 knockdown fetal AM hMSCs restored their ability to undergo cardiomyogenesis (1.6% ± 0.4%, n = 2500) in coculture with neonatal myocytes. Gap junctional coupling is required for differentiation of fetal AM hMSCs into functional CMs
Hou et al. (2013)	Rat BM-derived MSCs	BMP-2 and 5-azacytidine treatment	Combined treatment with BMP-2 and 5-AZA significantly improved the cardiac differentiation with fewer cell damage effects as compared to either of the them alone, and combined treatment was safer and effective method of induction in vitro. Expression of cTnI and Cx-43 was used as differentiation markers
Liu et al. (2013)	Rat BM-derived MScs	Combined treatment with 5-AZA (10 μmol/L), Ang-II (0.1 μmol/L), PFT-α (20 μmol/L), and BMP-2 (10 μg/L)	Development of cardiomyocyte-like cells expressing cTnT, cTnI, and Cx43 suggesting that the combination of inductors improved rate of differentiation. Total potassium current level

(continued)

Table 1 (continued)

Author/year	Cell type	Method of differentiation	Main findings
			and calcium transient in PFT-α cardiomyocyte-like cells was also higher in the differentiated cells
Szaraz et al. (2017)	BM-derived MSCs and UC-perivascular cell MSCs	Coculture on feeder layer	Increased expression of cardiomyocyte markers (i.e., MEF2C, cardiac troponin T, heavy chain cardiac myosin, signal regulatory protein α, and connexin 43) showed aggregate-based contracting cells in vitro

ANP atrial natriuretic peptide, *BNP* brain natriuretic peptide, *BM* bone marrow, *CMCs* cardiomyocytes, *cTnT* cardiac troponin T, *Cx-43* connexin 43, *MEF2c* myocyte enhancer factor 2C, *FACS* fluorescence-activated cell sorting, *MSCs* mesenchymal stem cells.

for clinical use of the cells (Trivedi et al. 2019). In the published data, MSCs have also been reported as mesenchymal stromal cells, mesenchymal progenitor cells, multipotent mesenchymal stromal cells, BM stromal cells, BM-derived MSCs, multipotent stromal cells, mesenchymal precursor cells, and medicinal signaling cells (Caplan 1991; Bobis et al. 2006; von't Hof et al. 2007; Deng et al. 2008; Samsonraj et al. 2017).

MSCs are a heterogeneous group of cells that constitute only 0.001–0.01% of the stem/progenitor population in the BM (Haider 2006; Wilson et al. 2019). They, therefore, necessitate extensive amplification in vitro culture to achieve a sufficient number for cell therapy applications (Lennon and Caplan 2006). Although MSCs were first isolated from the BM more than 50 years ago (Friedenstein et al. 1970), there are no unique surface markers for their definite identification from other cells (Haider 2018). More recently, the International Society for Cell Therapy (ISCT) has suggested standardized criteria to define human MSCs, which includes (1) adherence to the plastic surface under standard culture conditions; (2) the expression of CD73, CD90, and CD105 and lack of CD34, CD45, HLA-DR, CD14 or CD11b, and CD79a or CD19 membrane surface molecules; and (3) tri-lineage differentiation potential to adopt osteoblasts, adipocytes, and chondroblasts under a defined set of culture conditions in vitro. The ISCT standardized norms for the characterization of MSCs have been instrumental in removing the inconsistencies regarding the nomenclature as well as biological characteristics of MSCs. It is important to mention that MSCs from different species and even from various tissue sources cultured under a different set of conditions may differ in the expression of surface markers (Boxall and Jones 2012; Jones and Schäfer 2015) as well as in the number of isolated cells (Sullivan et al. 2015; Yoshimura et al. 2007) and their efficacy (Shariatzadeh et al. 2019). Moreover, the innate expression levels of a set of surface markers are not a guarantee of MSC homogeneity.

Besides other cell types, MSCs are an integral part of the HSC niche in the BM and essentially instrumental in offering a unique microenvironment for the HSCs

(Morrison and Scadden 2014). Together with the endothelial cells and megakaryocytes, MSCs provide proximity to the HSCs and contribute to the maintenance of niche homeostasis by providing instructive cues for their quiescence and functional activity (Schepers et al. 2015; Asada et al. 2017). These MSCs have been identified as CD45-Nestin+, contain all CFU-fibroblastic activity associated with HSCs, and respond to adrenergic stimulation (Méndez-Ferrer et al. 2010).

Isolation and Characterization of MSCs

Other than the BM, MSCs have been isolated from many other adult- as well as fetus-associated tissues, including adipose tissue, peripheral blood, lung, marrow spaces of a long bone, synovial fluids, muscle, placenta, umbilical cord, cord blood, periodontal ligaments, and dental pulp (Aust et al. 2004; Smiler et al. 2008; He et al. 2007; Griffiths et al. 2005; Tuli et al. 2003; Fan et al. 2009; Gay et al. 2007; Jackson et al. 2010; Anker et al. 2004; Miao et al. 2006; Corrao et al. 2013; Ong et al. 2014; Erices et al. 2000; Mareschi et al. 2001; Camilleri et al. 2016) (Fig. 1).

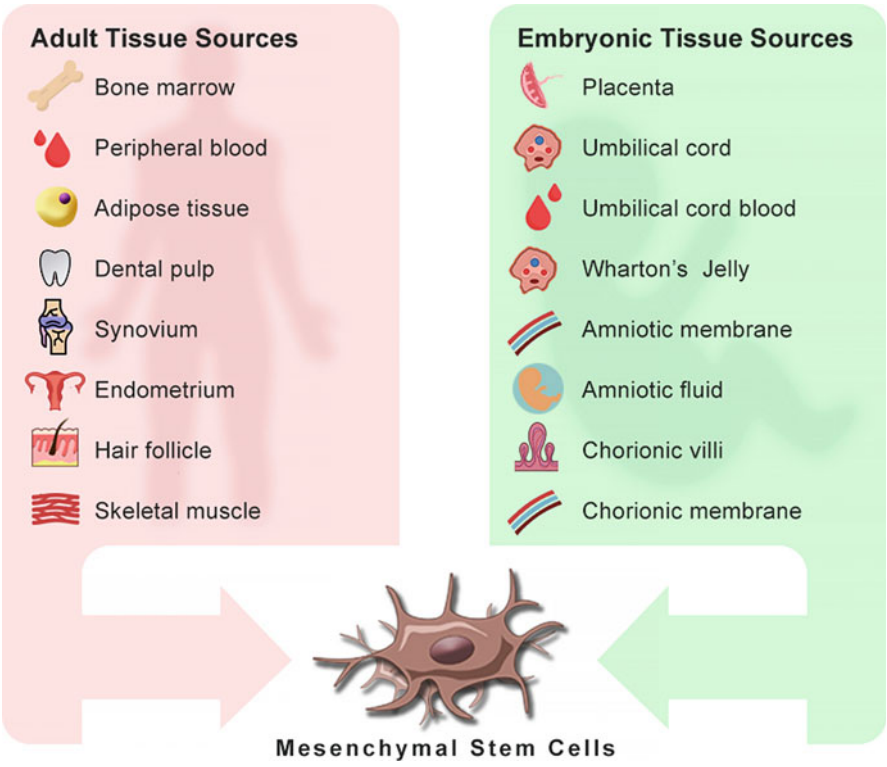

Fig. 1 Some of the adult- and fetus-associated tissue sources of MSCs used in experimental or clinical studies

The lineage-tracing studies strongly propose that progenitor cells of the MSCs come from around the blood vessels (capillaries, arteries, and veins), thus pointing their perivascular origin (Seo et al. 2004; Corselli et al. 2010).

Besides other parameters, the quality of MSC preparation is determined by their isolation procedure and expansion in the culture conditions (Haider 2018). The three steps needed for isolation and purification of MSCs from the BM include their separation from the nonnucleated RBCs by density gradient centrifugation, adherence to the plastic surface, and removal of monocytes by trypsinization. Generally, mechanical and enzymatic dissociation of the tissue has their respective advantages, but combining both of the approaches may enhance the yield of MSCs as compared to the enzymatic digestion alone as it results in loss of extracellular matrix that increases their time of adherence and low yield (Mushahary et al. 2018). Expansion of plastic adherence is the most employed way of obtaining and expanding the MSCs. Given their 3D habitat in the niche, the 2D in vitro culture expansion of MSCs may lead to loss of their progenitor potency and function. A paradigm shift in MSC culture in vitro uses 3D culture conditions that closely mimic their natural habitat (Hoch and Leach 2014).

Besides improving their proliferation rate, paracrine activity, and differentiation potential, more recent studies have shown that 3D culture conditions promote the expression of pluripotency genes (Zhou et al. 2017). Elucidating the underlying mechanism, it has been attributed to the relaxation of the cytoskeleton due to more conducive culture conditions. The commonly employed 3D methods include the hanging-drop approach, scaffold-free method, spin or rotate wall vessels, and fabricated membrane culture methods (Bartosh et al. 2010; Zhang et al. 2015; Miyagawa et al. 2011).

As discussed earlier, MSCs are generally characterized by their capacity to form colonies, renew themselves, express surface markers, and differentiate into multilineages (Friedenstein et al. 1974). The MSC colonies show heterogeneous morphological characteristics ranging from fibroblastoid- to spindle-shaped or from large-flattened to small-round cells. The first recognized multipotent stromal precursor cells from the BM were the colony-forming unit-fibroblastic (CFU-F), which are mainly composed of primary BM-derived MSCs. After further proliferative expansion in culture, they constitute MSCs/stromal cells, and in vitro they give rise to colonies during their initial growth (Pittenger et al. 1999). CFU-F is the efficiency of self-renewal assessed by the rate of colony formation that is a routinely employed standard approach to characterize MSCs.

Additionally, research labs from all over the world have diverse sets of antigens for characterization, and there is no consistency in the use of cell surface antigens for the isolation of MSCs. There is no one universal marker that precisely identifies MSCs. This divergence in surface marker expression besides extremely low-frequency presence in the tissues renders it challenging to identify MSCs in vivo. From among the wide array of surface antigens expressed by MSCs, CD105, CD73, and CD90 are reckoned as the primary markers, which are expressed on more than 95% of MSCs, while the expression of CD105, CD90, and CD73 is not completely specific to undifferentiated multipotent MSCs as some of these markers are also expressed by vascular cells, smooth muscle cells, and mature stromal cells

such as fibroblasts (Dominici et al. 2006). On the contrary, cultured MSCs do not express CD45, CD34, CD14, CD11b, CD19, and HLA-DR. Additionally, MSCs expressing STRO-1, CD146, SSEA-4, CD271 (NGFR), and MSC antigen 1 (MSCA-1) have been identified but with little significance as markers of identification and purification (Andersen et al. 2011; Jones et al. 2006). Nestin, a neural stem cell marker, has also been reported as a selective marker for BM-MSCs (Nombela-Arrieta et al. 2011; Méndez-Ferrer et al. 2009, 2010).

Important Characteristics of MSCs

Differentiation Potential of MSCs

Friedenstein was the first researcher who described that MSCs could differentiate into mesodermally derived cells (Friedenstein et al. 1974). Henceforth, it was shown that MSCs can cross lineage restriction and transform into morph functionally competent osteogenic, adipogenic, chondrogenic, vasculogenic, and myogenic phenotypes (Piersma et al. 1985; Caplan 1986; Wakitani et al. 1995; Kopen et al. 1999). This inherent property of multipotentiality may be accentuated in the presence of various factors such as ascorbic acid, dexamethasone, bone morphogenetic proteins (BMPs), WNTs, fibroblast growth factors (FGFs) besides other heparin sulfate-sensitive morphogens, and growth factors (Bhakta et al. 2012; Bramono et al. 2012; Dombrowski et al. 2013; Helledie et al. 2012; Ling et al. 2010; Teplyuk et al. 2009). While dexamethasone, indomethacin, insulin, and isobutylmethylxanthine generally support adipogenic differentiation (Scott et al. 2011), the presence of ascorbate, insulin, transferrin, selenic acid, and TGF-β promotes chondrogenesis (Johnstone et al. 1998; Mackay et al. 1998; Barry et al. 2001). Similarly, treatment with 5-azacytidine induces the cardiomyogenic differentiation of BM-derived MSC in vitro (Makino et al. 1999; Fukuda 2001; Antonitsis et al. 2007; Ullah et al. 2021). Please refer to summary of studies reporting in vitro differentiation of MSCs (Table 1).

Other factors included in the culture medium, i.e., antibiotics, serum, and growth supplements, to support cell stability and proliferation may significantly interfere with the undifferentiated expansion of MSCs (Riis et al. 2016; Pountos et al. 2014; Gharibi and Hughes 2012; Lee et al. 2001). On the same note, cellular and subcellular level preconditioning approaches have been developed to promote survival signaling, cell proliferation, differentiation, and paracrine activity of the preconditioned cells (Haider et al. 2010b; Lu et al. 2010, 2012; Suzuki et al. 2010; Haider and Ashraf 2010; Kim et al. 2009; Afzal et al. 2010). More recently, the focus has shifted to the use of pharmaceutical-grade human plasma derivatives and platelet lysate to promote MSC proliferation (Diez et al. 2015; Haider 2017). In vitro expansion of MSCs is also imperative during the use of autologous cell therapy due to the limited availability from the tissue source, especially from the aging patients as aging impairs their proliferation and differentiation potential (Kretlow et al. 2008).

Haider and colleagues have already reported that the doubling time of BM-derived MSCs from aging rats (24 months of age) was significantly higher than the young donor MSCs (Jiang et al. 2007, 2008; Haider et al. 2008). The harvest of MSCs was meager, and it took a significantly more extended time for the aging BM cells to adhere to the plastic surface. Moreover, when the cells got adhered to the plastic surface, they were stickier and required longer time to get dislodged from the tissue culture plates during trypsinization. In an attempt to support their proliferation in vitro culture, Igura and colleagues have reported that transgenic overexpression of neuropeptide Y (NPY) receptors and subsequent treatment with NPY5 significantly enhances MSCs' rate of proliferation (Igura et al. 2011). Other strategies to enhance MSC proliferation include concomitant transgenic overexpression of Ang-1 and Akt with downstream involvement of miR-143, a critical regulator of cell proliferation (Lai et al. 2012). One needs to understand that culture-expanded colonies of MSCs show limited differentiation potential that ranges from bi-potency (osteogenic and chondrogenic lineages) to monopotency (Muraglia et al. 2000; Pevsner-Fischer et al. 2011; Russell et al. 2010). The reason for this variation is not well understood, but it may be due to the epigenetic adaptations to the culture conditions.

Trophic Functions of MSCs

Various stem cell research groups now support the "paracrine hypothesis" and attribute the therapeutic benefits of MSCs with their capability to release trophic factors with or without their ability to undergo differentiation (Lei and Haider 2017). MSCs are also capable of generating a reparative microenvironment with their paracrine secretions rich in bioactive molecules, including chemokines, cytokines, morphogens, growth factors, microvesicles, and microRNAs (Kordelas et al. 2014; Lai et al. 2010; Amable et al. 2014; Eirin et al. 2014, 2016; Haider and Aramini 2020). Besides vasculogenic and myogenic differentiation potential and immune-regulatory properties, paracrine activity is an essential characteristic of MSCs that tips them as one of the choice cells for cell-based therapy.

Various research groups have provided evidence that the MSCs can maintain the growth, viability, and multipotent status of HSCs in long-term cocultures which lacked growth factor supplementation by secreting these trophic factors (Spees et al. 2016; Dexter et al. 1977; Dexter and Spooncer 1987; Queensberry et al. 1989; Wu et al. 2013). Although the paracrine hypothesis has gained popularity and acceptance as one of the important underlying mechanisms of cell therapy and has led to the novel strategy of cell-free therapy (Haider and Aziz 2017), there is no comprehensive uniform list of paracrine factors released from MSCs. The paracrine activity of MSCs is sensitive to the signals from their microenvironment. Hence, the composition of the paracrine secretions is influenced by a multitude of factors, including physical factors, i.e., hypoxia, stretch, pulsed focused ultrasound, electrical stimulation, and heat-shock treatment (Kusuma et al. 2017; Lei and Haider 2017; Antebi et al. 2018; Razavi et al. 2020; Parate et al. 2020), and chemical cues, i.e., vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), and IL-6

(Jeanmonod et al. 2018; Wei et al. 2013). Haider and colleagues have already reported that exposure to intermittent cycles of anoxia-reperfusion during preconditioning of the cells significantly altered their paracrine activity (Kim et al. 2009, 2012a). A marked upregulation of various growth factors and HIF-1α-related miRs including miR-107 and miR-210 in the preconditioned cells was observed (Kim et al. 2012b). Besides physical and chemical manipulation, MSCs have also been genetically modulated to accentuate their paracrine activity (Jiang et al. 2006; Haider et al. 2008; Ahmed et al. 2010; Haider et al. 2011).

Immunomodulatory Characters of MSCs

Bartholomew and colleagues were the first to describe the ability of allogenic MSCs to modulate immune responses in a baboon model of skin allograft (Bartholomew et al. 2002). Immunomodulatory characters of MSCs may be due to their hypoimmunogenic nature, ability to alter the T-cell response, and immunosuppression of the local microenvironment while modulating angiogenesis, apoptosis, and cell proliferation (Atoui and Chiu 2012; Faiella and Atoui 2016; Hong et al. 2012; Marfy-Smith and Clarkin 2017). Although the immune-regulatory properties of MSCs remain less well defined, the general perception is that MSCs can modulate immune cells' functions by cell-to-cell contact and by secretory mechanisms involving the paracrine release of bioactive molecule factors. Moreover, MSCs express low levels of major histocompatibility complex (MHC) class I and lack MHC class II and co-stimulatory molecule B7 and CD40 ligand (Devine and Hoffman 2000; Majumdar et al. 2003; Tse et al. 2003). They can also interfere with the normal B-cell function by the T-cell suppression (Castro-Manrreza and Montesinos 2015). Besides their anti-apoptotic and anti-inflammatory functions, MSCs interact with tumor cells via paracrine signaling to increase the risk of metastasis (Costanza et al. 2017; Lacerda et al. 2015; Ye et al. 2012; Ridge et al. 2017).

Preclinical Studies with MSCs

Small Experimental Animal Studies

MSCs are genetically stable and less susceptible to malignant transformation (Izadpanah et al. 2006). They have been extensively characterized in various small and large experimental animal models for safety and reparability. Since the early report of heart cell therapy using lineage-negative (Lin-) BMCs expressing enhanced green fluorescent protein (eGFP) in a mice model of coronary artery ligation, it was observed that the transplanted cells repopulated the infarcted myocardium with neomyocytes by 9 days after treatment. The neomyocytes were also interspersed by vascular structures, a which contributed to the recovery of global cardiac function (Orlic et al. 2001). Similar observations were later reported in a study which used human BM-derived MSCs for transplantation in an adult

murine heart (Toma et al. 2002). These encouraging data lead to a plethora of experimental studies in vitro as well as in the preclinical settings to support the safety and effectiveness of BM-derived MSCs for myocardial repair and paved the way for their further investigations in the human patients.

A direct comparison of BM-derived MSCs and MNCs in a porcine model of chronic IHD showed superiority of the former over the latter cells in terms of improvement of systolic function (van der Spoel et al. 2015). A meta-analysis of the translational studies shows that BM-derived MSC-based cell therapy is safe and effective in preserving ischemic heart function including LVEF (van der Spoel et al. 2011). The proposed mechanism is multifactorial and involves stimulation of angiogenesis and neovascularization (Psaltis et al. 2008; Haider et al. 2008), neomyogenesis (Nagaya et al. 2004; Haider 2006), paracrine effects (Mirotsou et al. 2007), reduction of fibrosis (Molina et al. 2009; Jin et al. 2020), immunomodulation (Van den Akker et al. 2013; Hamid and Prabhu 2017), and stimulation of endogenous cardiac stem cells (CSCs) to proliferate and participate in the repair process (Hatzistergos et al. 2010; Table 2).

Large Animal Experimental Models: Translational Studies

Large experimental animal studies are considered a critical step forward in establishing novel treatment approaches for diseases. The data generated from these studies are more relevant for translation to humans and hence greatly influence the advancement of novel treatments to the clinical phase of assessment. Given that stem cell-based therapy has already entered into the clinical phase of evaluation for cardiovascular applications, the support from the large experimental animal models is of utmost significance in translating the continuum of safety and efficacy data for a rationale designing the clinical trials (Harding et al. 2013). However, the need for large animal models has been generally ignored by the researchers due to their high cost, complexity, labor-intensive nature, and less suitability for the mechanistic understanding of cell-based therapy. Amid these challenges, which have restricted them to less than optimal usage to treat specific cardiac pathologies, the debate for an ideal translational model for modeling of cardiac pathologies continues unabated. However, as a fundamental principle, the body-to-heart weight ratio of the experimental animal should be comparable to that of humans to yield reliable simulation besides giving due consideration to other factors such as age appropriateness, etc. (Milani-Nejad and Janssen 2014; Haider 2018).

A wide array of large experimental animal models, i.e., pigs, dogs, sheep, monkeys, etc., have been developed to model CVDs in general and assess stem cells' safety and regenerative potential. However, experimental pig models of myocardial injury have been preferred over the other models and are primarily classified as open and closed-chest models with their respective advantages and limitations (Munz et al. 2011). Some of the typical translational studies using stem cells for myocardial repair and regeneration and their outcome have been summarized in Table 3.

Table 2 Summary of preclinical trials utilizing BM-derived MSCs in the small animal models of heart diseases

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
Wang et al. (2006)	Allogenic BM-MSCs with or without VEGF overexpression (^{VEFG}MSCs)	Intramyocardial injection of ^{VEGF}MSCs and cytokine therapy	48	AMI	Mice	Extensive regeneration and survival of ^{VEGF}MSCs in and around the infarcted heart. Blood vessel density significantly increased in ^{VEGF}MSCs as compared to other groups in peri-infarct area. Fibrosis was significantly reduced with improved LV contractile function. LV systolic and diastolic functions were well preserved in ^{VEGF}MSCs as indicated by +dP/dt, -dP/dt, and Tau (glantz)
Noiseux et al. (2006)	Allogeneic	Intramyocardial injection	12	AMI	Mice	MSC engraftment within infarcted myocardium was transient but significantly enhanced by Akt-MSCs fusion with CMs and was observed but was infrequent. Also a low rate of cardiomyogenic differentiation of MSCs was observed. MSC-Akt decreased infarct size at 3 days and restored early cardiac function. These observations confirmed that paracrine mechanisms mediated by MSC are responsible for CM survival and Akt could alter the secretion of various cytokines and growth factors

(continued)

Table 2 (continued)

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
Boomsma et al. (2007)	Syngeneic	Intravenous injection	25	AMI	Mice	Two weeks after coronary occlusion, there was a significant drop in LV systolic pressure, dP/dt (max), dP/dt(min), ESPVR, and E (max) and a significant increase in end-diastolic volume in vivo. Femoral vein injection of MSCs 1 h after occlusion attenuated the cardiac dysfunction without altering infarct size or end-diastolic volume. Injected MSCs pre-labeled with fluorescent paramagnetic microspheres were observed scattered in non-infarcted myocardium. Flow cytometry of whole heart digests after intravenous injection of MSCs labeled with either fluorescent microspheres or fluorescent PKH26 dye showed that infarcted hearts from mice that received MSC injections contained significantly more cells that integrated into the heart than those from non-infarcted controls
Derval et al. (2008)	Allogeneic	Patch system	107	Chronic	Murinae	This study showed that cell therapy improved angiogenesis and cell survival in the scar but only MSCs exhibited the capacity to invade the

						scar. No evidence of myocardial or vascular regeneration from progenitor cells. Engraftment of the progenitors/unfractionated BMC mix increased repopulation and thickness of the scar
Dai et al. (2008)	GCSF mobilization of and intramyocardial injection of BM-MPCs	GCSF mobilization of BM cells	96	AMI	Mice	LVEF and LVFS improved in MPC-treated animals as compared to control animals which received the medium. The infarcted area was also significantly smaller in MPC transplanted hearts than that in control hearts. MPC injection significantly promoted GFP+ cells homing from BM
Jin et al. (2011)	First and fifth passage naïve BM MSCs	Intramyocardial injection	45	AMI	Mice	LacZ or β-gal in the hearts from the MI + first passage MSC group was greater, leading to increased LV wall thickness, significant reduction in fibrotic area, and significantly improved LVEF than MI + fifth passage BM-MSC group on week 6 after cell transplantation
Huang et al. (2013)	BM-MSCs overexpressing Mir-1, naïve, or null vector transduce MSCs	Intramyocardial injection	80	AMI	Mice	The transplanted MSCs were able to differentiate into cardiomyocytes in the infarcted zone. Cardiac function in the MSCs, MSCs-null, and MSCs-miR-1 groups was significantly improved compared to the PBS group ($p < 0.01$ or $p < 0.001$).

(continued)

Table 2 (continued)

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
						However, most significant improvement highest rate of myocyte differentiation was observed in miR-I-expressing MSCs
Liao et al. (2017)	Allogenic	Intravenous injection	54	N/A safety study	Mice	Clinical doses of BM-derived MSCs induced mild and reversible coagulation, which increased BM-derived MSC lung embolism and clearance. Anticoagulation treatment by heparin (400 U/kg) prevented BM-derived MSC-induced coagulation and the acute adverse effects of large-dose BM-derived MSC infusion efficiently. Heparin treatment decreased BM-derived MSC lung embolism and enhanced migration and maintenance of BM-derived MSCs to target organs in cell therapy. Based on an experimental colitis model, it was confirmed that heparin treatment enhanced the effect of BM-derived MSC therapy efficiently to reduce mortality, prevented weight loss, suppressed inflammation reaction, and alleviated tissue injury

Chen et al. (2020)	AD-MSCs	Tailorable hydrogel (ColT gel) or intramyocardial	150	AMI	Mice	The Col-Tgel created a suitable microenvironment for long-term retention of ADSCs in an ischemic area and enhanced their cardioprotective effects, significantly increased LVEF at 4 weeks after MI, and decreased LV end-systolic diameter but did not significantly decrease the LV end-diastolic diameter at 4 weeks after MI compared to PBS treatment
Barbash et al. (2003)	99mTc-exametazime labeled allogenic rat BM-MSCs	Intra-left ventricular injection vs. intravenous injection	39	2, 10, and 14 days after transient coronary artery occlusion	Rat	Comparison of intra-left ventricle, right ventricle, and intravenous routes. Drastically low uptake of the intra-left ventricular injected cells in the lungs and high uptake in the heart compared to intravenous injected cells. Histological evidence of cells in the infarct and peri-infarct regions on day 7 after intra-LV injection of cells
Davani et al. (2003)	Syngeneic	Intramyocardial injection	16	AMI	Rat	Thirty days after implantation, immunofluorescence studies showed engrafted cells expressing a smooth muscle phenotype (alpha SM actin+), as similarly observed in culture. Other engrafted cells lost their smooth muscle phenotype and acquired an endothelial phenotype (CD31+). Furthermore, vessel density was augmented in the MPC

(continued)

Table 2 (continued)

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
						group compared to the control group. After 30 days, echocardiography showed an improvement in LV performance in MPCs than the control group
Nagaya et al. (2004)	Syngeneic	Intravenous injection	70	AMI	Rat	The engrafted MSCs were positive for cardiac markers: Desmin, cTn-T, and connexin43. Some of the transplanted MSCs were positive for von Willebrand factor and vascular structures. Capillary density increased after MSC transplantation. Infarct size was significantly smaller in MSC group than control group (24+/−2 vs. 33 +/− 2%, $P < 0.05$). MSCs decreased LV end-diastolic pressure and increased LV maximum dP/dt (both $P < 0.05$ and control)
Imanishi et al. (2008)	Allogenic vs. syngeneic MSCs	Intramyocardial injection	119	AMI	Rat	Both syngeneic and allogeneic MSC transplantations were useful for AMI, increased VEGF, and blood vessel density. The donor MSCs disappeared rapidly but became a trigger of VEGF paracrine effect. One day after cell transplantation, transient increase in IL-1 beta and MCP-1 in recipient

						hearts were enhanced in allogenic MSCs, with macrophage infiltration at the injection site
Atoui et al. (2008)	Xenogeneic BM-MSCS or fibroblasts	Intramyocardial injection	90	AMI	Rat	Xenogeneic transplanted human BM-derived MSCs survived in the rat heart for more than 8 weeks without the use of immunosuppressants. The implanted MSCs significantly contributed to the improvement in ventricular function and attenuated LV remodeling. No immune cell infiltration, characteristic of immune rejection, was noted in MSC transplanted cells
Haider et al. (2008)	Allogenic rat MSCs with or without IGF-1 overexpression ($^{IGF\text{-}1}$MSC)	Intramyocardial injection	78	LAD ligation//AMI	Rat	Elevated myocardial IGF-1, pAkt, and SDF-1 in $^{IGF\text{-}1}$MSCs. Massive mobilization and homing of ckit+, MDR1+, CD31+, and CD34+ in $^{IGF\text{-}1}$MSCs. Extensive angiomyogenesis in the infarcted heart with significantly improved LVEF and LVFS
Enoki et al. (2010)	Allogeneic	Intramyocardial injection	40	Chronic	Rat	One month after MI, rat hearts were injected with MCs in the presence or absence of 10 μg/ml IGF-1 with or without PI3K inhibitor, 5 μM LY294002. IGF-1 significantly increased engraftment of MSCs between 6 h and 3 days after transplantation associated with the increase in stromal cell-derived

(continued)

Table 2 (continued)

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
						factor-1α in the infracted LV. IGF-1-MSCs significantly increased neovascularization and inhibited CM apoptosis 3 days and 1 month after MSC transplantation. This improved LV function 1 month after MSC transplantation. LY294002 abrogated all of the beneficial effects of MSC transplantation with IGF-1
Kim et al. (2012a)	Allogenic MSC overexpressing mir-210 or preconditioned	Intramyocardial injection	48	AMI	Rat	Induction of miR-210 in MSCs, preconditioned or transfected, promoted cell survival post-engraftment in the infarcted heart. Moreover, direct transfer of pro-survival miR-210 from miR-MSCs to host cardiomyocytes led to functional recovery of the ischemic heart. Improved LVEF, attenuated infarct size, and reduced fibrosis
Armiñán et al. (2010)	Human cells (xenogenic engraftment)	Intramyocardial injection	90	AMI	Rat	Both cell types induced an improvement in LV cardiac function and increased tissue cell proliferation in myocardial tissue and neoangiogenesis. However, MSCs were more effective for the reduction of infarct size and prevention of ventricular

						remodeling. Scar tissue was 17.48 +/− 1.29% in the CD34 group and 10.36 +/− 1.07% in the MSCs group ($p < 0.001$ in MSCs vs. CD34). Moreover, unlike MSC, CD34 (+)-treated animals showed local inflammatory infiltrates in LV wall that persisted until 4 weeks after transplantation
Li et al. (2012)	Allogeneic	Intramyocardial injection/intravenous injection	36	AMI	Rat	The cardiac function of the intramyocardial group increased significantly compared to the intravenous and control groups at day 7. In intramyocardial group, labeled cells were visualized in the infracted heart by serial MRI, and the intensity by OI was significantly higher on day 1. In the intravenous group, the heart signals were significantly attenuated by dual-modal tracking at two time points
Wang et al. (2012)	Allogenic	Intramyocardial injection	18	AMI	Rat	Every week for 6 weeks, hearts from three rats injected with MSCs were harvested to observe single CMs. Although each week numerous round MSCs were found in the hearts of animals treated with MSCs, beating CM-like cells labeled with PKH26 were observed by the sixth week of observation. The contractility of CM-like cells

(continued)

Table 2 (continued)

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
						was the same to that of the unlabeled contractile native CMs at the sixth week and that of the normal group (10.71 ± 1.59 vs. 11.09 ± 3.42 vs. 11.21 ± 2.16, $p > 0.05$). The contractility of CM-like cells was greater than cells both from the first week (10.71 ± 1.59 vs. 7.37 ± 3.47, $p < 0.01$) and the second week (10.71 ± 1.59 vs. 8.08 ± 3.11, $p < 0.05$) which was associated with significantly increased LVEF
Flynn et al. (2012)	Allogenic	Intramyocardial injection	17	AMI	Rat	Assessed 28 days after MI, the delivery of MSCs 24 h post-MI did not improve LVEF ($p = 0.19$) and did not prevent the decline in LVEF observed in the absence of cell therapy ($p = 0.17$). The administration of unrestricted somatic stem cells also did not improve LVEF ($p = 0.11$) but prevented a further decline in LVEF ($p = 0.001$). The infarct area ($p = 0.2$), apoptosis ($p = 0.07$), and angiogenesis ($p = 0.09$) did not differ between groups

Chen et al. (2019)	Allogenic	Intramyocardial injection	14	AMI	Rat	Coculture showed that IL33-MSCs reduced T-cell proliferation and enhanced CD206+ macrophage polarization. Echocardiography showed LVEF was enhanced in IL33-MSC-injected rats compared with vector-MSC-injected rats. Postmortem analysis of rat heart tissue showed reduced fibrosis and less inflammation in IL33-MSC-injected rats
Zhu et al. (2009)	Skeletal myoblasts or BM-MSCs	Intra-arterial (LAD)	45	AMI	Rabbit	Four weeks after the cell transplantation, LVEF was significantly improved in all cell treatment groups compared to control. Neovascularization was observed in both of the treatment groups. There was no significant difference between the two cell types
Xu et al. (2010)	Allogeneic	Intravenous	46	AMI	Rabbit	The cardiac improved significantly in the US + microbubble + MSC treatment group. The number of capillaries stained by HE in US + microbubble + MSC group was much greater than that of the other groups. US + microbubble-mediated supply of MSCs increased the level of VEGF in ischemic myocardium

(continued)

Table 2 (continued)

Author/year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
Rahbarghazi et al. (2014)	Autologous	Intramyocardial	42	AMI	Rabbit	Transplanted GFP-positive MSCs were enriched with time in the peri-infarct border zone with differentiation potential into three major cell types of the heart, including cardiomyocytes, endothelial cells, and smooth muscle cells, and there was significant augmentation of microvascular density
Tanaka et al. (2016)	BM autologous preconditioned (2% oxygen culture) MSCs	Cell sheet-based delivery	N = 5 per group	Chronic MI	Rabbit	Preconditioned autologous BM-derived MSC sheets were implanted into a rabbit old MI model. Implantation of BM-derived MSC sheets increased angiogenesis in the peri-infarcted area and decreased the infarcted area, leading to LVEF improvement. Importantly, the therapeutic efficacy of the preconditioned BM-derived MSC sheets was higher than that of standardly cultured sheets

ADSC adipose-derived stem cells, *AMI* acute myocardial infarction, *BM* bone marrow, *BMC* bone marrow concentrate, *CMs* cardiomyocytes, *ESPVR* end-systolic pressure-volume relationship, *GCSF* granulocyte colony-stimulating factor, *LV* Left ventricle, *LVEF* left ventricular ejection fraction, *LVFS* left ventricular fractional shortening, *MPC* mesangiogenic progenitor cells, *MSCs* mesenchymal stem cells, *VEFG* vascular endothelial growth factor

Table 3 Summary of preclinical trials utilizing BM-derived MSCs in the large animal models of heart diseases

Author/ year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
Shake et al. (2002)	Autologous MSCs	Intramyocardial injection	14	Chronic	Porcine	Extensive survival and engraftment of MSCs at the site of cell graft. Expression of muscle-specific proteins after 2 weeks. Contractile dysfunction attenuated at 4 weeks in cell-treated animals as compared to controls. Attenuation of LV remodeling
Amado et al. (2005)	Allogenic MSCs	Intramyocardial injection	14	Acute	Porcine	Significant long-term retention of the injected cells at 2 months after delivery. Gross pathology showed significant reduction in fibrosis and near normalization of systolic and diastolic cardiac function as compared to the placebo-treated animals
Quevedo et al. (2009)	Allogenic	Transendocardial injection	10	Chronic	Porcine	Long-term MSC survival, engraftment, and tri-lineage differentiation after transplantation into chronically scarred myocardium. MSCs showed capacity for cardiomyogenesis and vasculogenesis which contribute, at least in part, to their ability to repair chronically scarred myocardium
Schuleri et al. (2009)	Autogenic	Surgical-anterior thoracotomy	15	Chronic	Porcine	Autologous MSCs' safety and efficacy in a heart failure model. Showed substantial structural and functional reversal remodeling. Support clinical trials of MSC therapy

(continued)

Table 3 (continued)

Author/ year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
						in patients with chronic ischemic cardiomyopathy
Schuleri et al. (2011)	Allogeneic	Myocardial injections	22	Chronic	Porcine	MSC-based therapy led to reduction in infarct size, whereas infarct size increased in non-treated animals
Cai et al. (2016)	Autologous	Intramyocardial injection	24	Acute	Mini-swine	Twenty animals survived full length of experiment. PET-CT showed better 18F-FDG uptake in the cell-transplanted LV segments. Improved global cardiac function. Molecular studies showed mTOR activation signaling and improved LV function due to paracrine activity
van der Spoel et al. (2012)	Autologous	Intracoronary, NOGA-guided transendocardial, or surgical	19	Acute	Porcine	Direct comparison of MSCs with BM-derived mononuclear cells and effect of repetitive injections. At 4 weeks, LVEF improved in MSC-treated animals, but endpoint analysis showed no significant difference in efficiency after surgical injections at 8 weeks
de Jong et al. (2014)	Allogenic	Intracoronary infusion	36 with moderate and 33 with severe infarct	Acute	Porcine	Encapsulated MSCs (eMSCs) transduced to express glucagon-like protein-1. Three incremental doses of eMSCs or Ringer's solution without cells. LVEF increased more (9.3%) in

						animals with severe infarction. Vessel density increased threefold in the infarct zone 30% more in the border zone
Sheu et al. (2015)	Autologous	Surgical	25	Acute MI	Porcine	Baseline LV injection fraction and LV chamber size did not differ among the five groups. By day 60, LVEF was highest in group 1 and lowest in group 2, significantly higher in group 5 than that in groups 3 and 4, and significantly higher in group 4 than that in group 3
Guadalupe et al. (2016)	Adipose tissue allogeneic MSCs overexpressing IGF/HGF	Intramyocardial injection	14	Acute MI	Porcine	Treatment with paMSC-IGF-1/HGF (1:1) vs. the other groups had a reduced inflammation in some sections analyzed. This also enhanced angiogenesis in ischemic myocardium. Although indices of cardiac function did not show significant improvement, cell retention and IGF-1 overexpression were confirmed within the myocardium
Lim et al. (2006)	Allogenic naïve or Akt-transduced MSCs	Intracoronary infusion	36	Acute MI	Porcine	Highest LV systolic function improvement and reduction in infarct size were observed by M-SPECT in Akt-transduced MSCs than naïve MSC- or vehicle-treated animals. Akt-MSCs survived better in the peri-infarct zone observed by FISH

(continued)

Table 3 (continued)

Author/ year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
Silva et al. (2005)	Allogeneic	Intramyocardial injection	20	Chronic	Canine	Mean LVEF was significantly higher in treated dogs at 60 days. WBC and CRP levels were similar in both groups. CK-MB and troponin-I increased from baseline to 48 h and then returned to baseline. There was a trend toward reduced fibrosis and more vascular density in treated group. MSCs co-localized with endothelial and smooth muscle cells but not with myocytes
Bartunek et al. (2007)	Autologous Pre-treated MSCs	Intramyocardial injection	30	Chronic	Canine	Pre-treated MSCs with cardiomyogenic factors significantly increased their myogenic differentiation and increased LV wall thickness by week 12. The biological ex vivo cardiomyogenic specification of adult MSCs is feasible and increases in vivo cardiac differentiation as well as the functional recovery
Mathieu et al. (2009)	Autologous	Intramyocardial injection	24	Chronic	Canine	Direct comparison of BM-derived MSCs and mononuclear cells. Treatment with latter were superior in giving sustained improvement in wall motion score index, reduction in infarct size, improved end-systolic elastance, and improved regional systolic function. VEGF-3 levels

						increased in border zone leading to more vascular density in mononuclear cell-treated animals
Wang et al. (2015)	Allogenic MSCs +bFGF	Retrograde coronary infusion	32	Subacute	Canine	Retrograde coronary venous bFGF infusion enhanced engraftment and differentiation capacity of MSCs, recovery cardiac function, improved LVEF, attenuated remodeling, and increased vascular density
Zhao et al. (2012)	Autologous	Surgical	10	Acute	Ovine	Significant attenuation of CM hypertrophy with the normalization of the hypertrophy-related signaling proteins PI3Kα, PI3Kγ, extracellular signal-regulated kinase (ERK), and phosphorylated ERK (p-ERK) in the adjacent zone of the MSC-treated group versus the MI-alone group
Locatelli et al. (2015)	Allogenic male donor naïve or VEGF-expressing BM-MSCs	Intramyocardial injection	24	AMI	Ovine	One month posttreatment with MSCs overexpressing VEGF, infarct size (magnetic resonance) decreased by 31% vs. pre-treatment. LVEF recovered to almost its baseline value and marked decrease in end-systolic volume. Moreover, the treatment induced significant capillary and arteriolar proliferation, which reduced subendocardial fibrosis. These data were in comparison with naïve MSC-injected animals

(continued)

Table 3 (continued)

Author/ year	Cell type	Mode of delivery	Sample size	Model type	Animal	Main findings
Rabbani et al. (2017)	Autologous	Intramyocardial injection	18	Acute	Ovine	Echocardiography revealed a significant improvement in the LVEF compared with the control group (p value <0.05). Vascular density increased in cell therapy groups. The electron microscopic evaluation of the infracted areas revealed cardiomyocytes in variable stages of development in the border zone
Hu et al. (2016)	Allogenic hypoxia cultured	Intramyocardial injection	49	Acute	Cynomolgus monkey	Direct comparison naïve and hypoxia-treated allogenic MSCs for long-term effects. Insignificant functional improvement between the two cell-treated groups. However, hypoxia-MSC transplantation was also associated with increases in cardiomyocyte proliferation, vascular density, myocardial glucose uptake, and engraftment of the transplanted

Quevedo and colleagues investigated the regenerative potential of BM-derived MSCs after long-term engraftment in a chronic myocardial infarction (MI) model (Quevedo et al. 2009). The cells were harvested through an iliac crest BM aspiration from a healthy male landrace pig, expanded in vitro, and labeled with 5-bromodeoxyuridine (BrdU). The experimental animal model of chronic MI was developed in female pigs by left anterior descending (LAD) occlusion for 150 min using an over-the-wire balloon catheter. The pattern of induced MI was consistently antero-apical in all of the experimental animals included in the study. Twelve weeks following the induction of MI, the animals were allocated to receive either a catheter-based transendocardial injection of placebo ($n = 4$) or male allogeneic BM-derived MSCs ($n = 6$). A noticeable decline in the LVEF, transmural extension of myocardial scar, and left ventricular remodeling was observed on a cardiac MRI scan at 12 weeks after the induction of MI. MSC-treated hearts showed almost one-third decrease in the infarct size (29%) 8 weeks after the intervention compared to the placebo-treated animals. Eulerian circumferential shortening (Ecc) was used to estimate the contractile function of the regenerated myocardium, which showed significant improvement in the border and infarct zones for up to 3 months of observation compared to the placebo-controlled group. Furthermore, a significant increase in the basal blood flow was observed as early as 4 weeks after BM-derived MSC therapy in the cell treatment group.

Schuleri and colleagues investigated the dose-response effects exerted by BM-derived MSCs on the infarcted myocardium (Schuleri et al. 2009). The experimental porcine model was developed by a temporary balloon occlusion of the LAD, similar to the approach described earlier by Quevedo et al. (2009). Ischemia was sustained for 120 min, followed by reperfusion by deflating the balloon. The BM-derived MSCs were harvested from the iliac crest of each animal and expanded in vitro for four to seven passages before transplantation. The cells were phenotypically characterized for CD45− and CD90+ expression besides the assessment for CFU-F. A total of 15 pigs were randomly assigned to one of the three arms of high-dose, low-dose, and placebo (saline) treatment. The cells were delivered by direct intramyocardial injections through an anterior thoracotomy into areas of severe hypo- and/ or akinesia at a rate of 15–25 injections per animal. A series of cardiac MRIs were obtained at different time intervals to assess the outcomes of the study. The study data revealed no observable safety and tolerability concerns (including neoplasms and arrhythmias) in all of the intervention groups. However, infarct size decreased significantly in both cell treatment groups (i.e., high and low dose) with a concomitant remodeling reversal when compared to the placebo-treated animals. Furthermore, the cell-treated animals had a significant enhancement in their cardiac contractile function and improved post-MI coronary basal flow and adenosine-dependent coronary flow reserve (CFR).

Building on these findings, Schuleri et al. conducted another study to investigate the usefulness of multi-detector computed tomography (MDCT) in assessing the therapeutic effects of myocardial regenerative cell therapies (Schuleri et al. 2011). They reported a massive 27.9% reduction in infarct size and a significant 13%

increase in LVEF. The authors concluded that MDCT was a valuable tool in evaluating infarct size and LVEF after intramyocardial cell delivery.

Cai et al. used an open-chest model of acute MI in mini-Chinese swine to elucidate the mechanism of stem cell-based improved cardiac function (Cai et al. 2016). Using PET-CT, the authors observed a significant increase in mean signal intensity (MSI) in the cell-transplanted hearts at 4 weeks as compared to their baseline levels at 1 week after cell transplantation. Gene expression of glucose transporters Glut1 and Glut4 and glucose metabolism-related enzyme phosphofructokinase was significantly increased in the cell-transplanted segments. The authors inferred that these molecular changes leading to global and regional left ventricular function resulted from the transplanted cells' paracrine.

Lim et al. previously used either naïve or genetically modified BM-derived MSCs overexpressing Akt (Lim et al. 2006). The sex-mismatched cells were delivered via intracoronary infusion 3 days after the development of experimental porcine model of MI developed by balloon occlusion for 30 min. Myocardial single-photon emission tomography (M-SPECT) revealed the highest LVEF improvement and reduced infarct size in Akt-MSC-treated animal hearts. Fluorescence in situ hybridization (FISH) for Y-chromosome revealed the highest survival of male donor cells in the peri-infarct zone in the female recipient animals. The authors observed the most significant cardiac function improvement in the Akt-MSC-treated animals and attributed it to the better donor cell survival. On the same note, de Jong et al. used genetically modified MSCs expressing glucagon-like peptide-1 (GLP-1) and microencapsulated them before transplantation in a porcine model of AMI (de Jong et al. 2014). One hundred animals were divided into posterolateral (moderate MI, $n = 36$) and anterior MI (severe MI $n = 33$) groups. The most significant LVEF improvement was observed in the GLP-1 eluting microencapsulated cells transplanted in severe MI animals. The microencapsulated cells served as a continuous source of GLP-1, an incretin hormone with cardioprotective effects. Moreover, there was a significant increase in vascular density in the infarct and peri-infarct areas in the animals treated with GLP-1-expressing MSCs.

Some of the other exciting studies in the porcine model include comparison of different routes of administration (Moscosoa et al. 2009; Gathier et al. 2019), percutaneous intramyocardial delivery of MSCs (Tao et al. 2015), validation of the use of contrast-enhanced MRI to monitor the efficacy of cell therapy (Malliaras et al. 2013), study of the effect of dose on the outcome of myocardial repair (Crisostomo et al. 2019), etc.

Despite their overwhelming abundance in the literature, the promising performance of BM-derived MSCs on improving the myocardial function in chronic ischemic models did not stop with swine models. For example, Silva et al. investigated the effectiveness of BM-derived MSCs in a canine model of chronic myocardial ischemia (Silva et al. 2005). Diverging from using an autologous source of the cells, Silva et al. utilized allogeneic canine cells. The cells were isolated and cultured using almost a similar protocol previously discussed and labeled with the cross-linkable membrane dye CM-DiI and the nuclear stain DAPI before transplantation. The chronic myocardial ischemia model was developed through a left thoracotomy

and placement of an ameroid constrictor over LAD. One month after ameroid constrictor placement, the animals were randomly divided to receive intramyocardial injection of allogeneic MSCs (treatment group, n = 6) or saline (control group, n = 6). Overall, the procedure was highly safe without any observed arrhythmias or pericardial effusions. Transthoracic echocardiography revealed a mean 10% reduction of LVEF in the control group. In comparison, it showed a significant 5% increase in the treatment group, with a concomitant reduction in fibrotic changes in the infarct area. Furthermore, a noticeable increase in the vascular density was detected in the BM-derived MSC treatment group. The authors claimed differentiation of Dil- and DAPI-positive cells into actin-positive neomyocytes leading to increased LV wall thickness.

On the same note, Zhao et al. investigated the effectiveness of allogenic BM-derived MSCs on cardiac remodeling using an ovine model of acute myocardial ischemia (Zhao et al. 2012). The cell preparation process followed a similar protocol as described earlier, and the purified cells were characterized by tri-lineage differentiation and flow cytometric analysis for surface marker expression to ascertain the purity of MSC preparation. Experimental MI was developed in ten male sheep through a surgical ligation of LAD and second diagonal coronary arteries, while another four healthy sheep were assigned to a sham group. The sham-treated group's hearts were not infarcted and did not receive any stem cell therapy. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to detect apoptotic cells. The total number of TUNEL staining nuclei was later used to estimate the nuclei undergoing apoptosis. Furthermore, hypertrophy-related signal proteins (e.g., PI3Kα, PI3Kγ, and p-ERK/ERK) were upregulated in the infarction zone in the animals that only received MI as compared to the sham group. Nonetheless, analysis of the identical zones in the BM-derived MSC treatment group revealed a normalized expression, almost indistinguishable from that of the sham group. Sonomicrometric analysis revealed a considerable decline in regional contractile function and remodeling strains in both cell treatment and control groups.

Throughout the years, various strategies have been adopted to enhance the efficacy of BM-derived MSCs. For example, van der Spoel et al. investigated the efficiency of different cell delivery methods in donor cell survival and therapeutic benefits (van der Spoel et al. 20,212). They compared intracoronary and transendocardial routes of delivery with the well-established surgical delivery in a chronic porcine model of ischemic cardiomyopathy. The BM-derived MSCs were radiolabeled with indium-111 (^{111}In) for later quantitative analysis of cell survival. The experimental animal model of MI was developed by median sternotomy induced in 24 healthy pigs by temporary ligation of the left circumflex artery for 75 min. The authors did not observe any significant difference in hemodynamic and echocardiographic parameters at the start of the trial and 1 month after the induction of MI. In addition, cell-related characteristics such as viability and count did not differ between the groups. However, intracoronary delivery showed more consistent results concerning the delivery efficiency, while higher levels of local retention (i.e., at the mid-papillary level) were observed in the animals receiving

transendocardial delivery. Noticeably, a high level (up to 45%) of ^{111}In-labelled cell retention was observed in extra-cardiac organs in all of the groups.

To enhance donor cell survival, various strategies have been adopted. In this regard, Sheu and colleagues investigated the effects of combining shockwave therapy and autologous BM-derived MSCs to improve the clinical outcome of the left ventricular failure (Sheu et al. 2015). Twenty-five male mini-pigs were equally divided into sham control (group 1), acute MI (AMI, group 2), AMI + shock wave (shockwave, group 3), AMI + autologous BM-derived MSCs (group 4), and AMI + shockwave + autologous BM-derived MSCs (group 5). The experimental animal model was developed by LAD ligation, and 1 hour later the animals received their respective treatment by intramyocardial injections. The trial results showed that dual therapy group receiving shockwave therapy and BM-derived MSCs showed a significantly higher LVEF improvement with a significant reduction in the infarct size and most wall thickness. Immunohistochemical staining revealed that the number of inflammatory indicators was also significantly lower in this group. Neovascularization in the infarct and peri-infarct area, as evident by the number of arterioles, was by far the highest in the AMI-shockwave BM-derived MSC group. These findings support the essential regenerative role of BM-derived MSCs and signify the anti-inflammatory role of shockwave therapy.

Although the use of BM-derived MSCs has already reached the veterinary clinics for various applications (Gomes et al. 2017), some other important studies using the canine model for myocardial repair include assessment of the efficiency of pre-treated MSCs with cardiomyogenic factors (Bartunek et al. 2007), comparison of BM-derived MSCs and mononuclear cells (Mathieu et al. 2009), histological evidence of the efficacy of allogenic MSCs in myocardial repair (Vela et al. 2009), combining cell delivery with growth factor treatment for myocardial repair (Wang et al. 2015), comparison of routes of administration for MSC delivery, using dual-isotope simultaneous acquisition (DISA) of SPECT for assessment (Perin et al. 2008; Hao et al. 2015), etc. Besides canine models, monkeys are also considered an excellent surrogate cardiac model (Yang et al. 2011) for translational assessment of stem cells for myocardial repair (Hu et al. 2016).

It is pertinent to add that that there is a wide array of experimental animal models, dominated by porcine models (Hotham and Henson 2020), which are available to study the safety and efficacy of stem cell-based therapy of heart diseases, and each one of these models has specific features (Ou et al. 2010; van der Spoel et al. 2011). These specific features could be exploited to add to the relevance of the study to achieve desired results for translation to humans and hence should be given due consideration before opting for a particular model (Gandolfi et al. 2011). For example, the presence of well-developed collateral circulation in the canine hearts is useful to study vascular adaptation to ischemic myocardial injuries and, therefore, may have an effect on the long-term benefits of stem cell therapy. Similarly, the ovine and porcine hearts, similar to humans', lack these adaptations, making them more suitable in studies where human-like anatomy is a required feature. However, the difficulties brought by the unique thoracic anatomy of ovine have limited its use due to technical challenges encountered during cardiac

imaging (e.g., transesophageal echocardiography). Moreover, ovine and porcine models may not be feasible for mechanistic understanding at molecular-level interplay involved in cell-based therapy. Despite its shortcomings, porcine models have gained huge popularity among researchers modeling heart disease for translational studies because of the many similarities swine heart shares with humans' in the aspects of gross anatomy, coronary circulation, and immunological and physiological responses (Cui et al. 2005; Milani-Nejad and Janssen 2014).

MSCs in the Clinical Perspective

Stem cell-based therapy has emerged as novel science that should be considered for the routine treatment of cardiac damage. MSCs are currently the most used cells for cell-based cardiovascular therapy (Rajab et al. 2020). Based on the safety and efficacy data from the preclinical and translational studies and given their ease of ability and biological characteristics, they have progressed to advanced phases of clinical assessment including several randomized placebo-controlled phase II and even phase III trials for different diseases (refer to Chapter-5, section 4 & Table-1 in this book). Despite the dominance of autologous MSCs in these clinical studies, the use of allogeneic MSCs has also gained popularity with encouraging results in general and for cardiovascular applications (Table 4). Irrespective of the tissue source for MSCs, the primary efficacy endpoint in these clinical trials has been to investigate if MSC transplantation could successfully improve the indices of LV structure and function in patients with IHD. The following section summarizes some of the randomized clinical trials involving BM-derived MSCs from autologous and allogenic sources to discuss the advantages and limitations of the cells from each source of cell in the clinic.

Randomized Clinical Trials with Autologous or Allogenic BM-Derived MSCs

The POSEIDON Trial

The significance of the POSEIDON trial (Clinicaltrials.gov Identifier: NCT01087996) is that it compares the safety and efficacy of autologous vs. allogenic BM-derived MSC-based cell therapy in treating myocardial damage (Hare et al. 2012). A total of 30 patients with ischemic cardiomyopathy were included in the study. Cardiac CT screening was done at 13-month follow-up for detecting LV function and volumes besides quality of life, physical health, LVEF, and other related cardiac measurements. Only one participant from each treatment group had SAE during 30-day follow-up after treatment and needed emergent hospitalization for heart failure. The 1-year incidence of SAE was 33.3% ($n = 5$) vs. 53.3% ($n = 8$) in the allogeneic vs. autologous groups ($p = 0.46$). Four patients suffered ventricular arrhythmias in the autologous group, while no ventricular arrhythmias were observed in the allogenic group. The trial data concluded safety and efficacy of stem cell therapy in cardiac patients in terms of functional status and

Table 4 Summary of clinical trials utilizing BM-derived MSCs in the management of heart failure

Author/year	Cell type	Mode of delivery	Sample size	Trial phase	Placebo	Primary outcomes	Findings
Mathiasen et al. (2020)	Autologous	I/M injection	60	2	Phosphate-buffered saline	Change in LVESV by MRI or CT	Significant reduction of LVESV in MSC group
Butler et al. (2017)	Allogenic	IV	22	2	Ringer's lactate	Safety and efficacy	Nonsignificant
Karantalis et al. (2014) (PROMETHIUS)	Both	I/M injection	9	Suspended (1 and 2)	No placebo group	12 months adverse events	Insufficient results
Heldman et al. (2014)	Both	Intraventricular injection with infusion catheter	65	1 and 2	Vehicle placebo	30-day adverse composite event	None (0% events)
Hare et al. (2012) (POSEIDON)	Both	Transendocardial stem cell injection	30	1 and 2	None	30-day emergent serious adverse events	6.7% rate (1 patient in each group)

quality of life improvement. However, there was no significant improvement in 6-min walk test (6MWT) in the allogenic group compared to the baseline, while no significant LVEF and LV end-diastolic volume changes were observed in the autologous group. Interestingly, low-dose allogenic MSC (20 million cells) treatment led to highest reduction in LV end-diastolic volumes. Lack of placebo arm is a limitation of this study, along with a small size of participants.

The same research group registered another trial POSEIDON-DCM (ClinicalTrials identifier: NCT01392625) comparing allogenic vs. autologous BM-derived MSCs in patients with dilated cardiomyopathy. A total of 37 patients were randomized in a ratio of 1:1 to transendocardially (TESI) receive 100 million autologous or allogenic MSCs using NOGA catheter (Mushtaq et al. 2014). For baseline, the patients were followed up on day 30, 3, 6, and 12 months for safety and efficacy endpoints including serious adverse events (SAE), LVEF, 6MWT, MACE, and immune-biomarkers. The authors did not observe any treatment-related SAE in both the treatment groups until day 30 but observed SAEs 28% (95% CI: 12.8, 55.1) in allogenic treatment group vs. 63.5% (95% CI: 40.8, 85.7; $p = 0.1004$) in autologous treatment group (Hare et al. 2017). LVEF was up by 8 units (95% Cl: 2.8, 13.2; $p = 0.004$) vs. 5.4 (95% Cl: −1.4, 12.1; $p = 0.116$, allogenic vs. autologous treatment $p = 0.4887$) units in allogenic vs. autologous treatment groups, respectively. Interestingly, MACE rate was also lower in allogenic treatment group ($p = 0.0186$ vs. autologous group) besides a significant decrease in tumor necrosis factor-alpha ($p = 0.00001$). These findings clearly showed the superiority of allogenic MSCs in the clinical perspective.

The PROMETHEUS Clinical Trial

Researchers at the University of Miami and Johns Hopkins University conducted a **Pro**spective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS; ClinicalTrials.gov Identifier: NCT00587990) (Karantalis et al. 2014). During the phase I/II randomized, double-blind, placebo-controlled study, a total of six patients received intramyocardial injections of autologous MSCs as an adjunct to coronary artery bypass grafting (CABG). The cells were injected in the akinetic/hypokinetic myocardium, which were not receiving bypass graft and assessed by cardiac MRI during follow-up at intervals of 3, 6, and 18 months. All the patients enrolled in this study had a known history of chronic ischemic LV dysfunction after a MI. Cardiac MRI at 18 month revealed increased LVEF ($9.4\% + 1.7$, $p = 0.0002$) and decreased scar mass($-47.5 + 8.1$, $p = 0.0001$) as compared with the baseline values. Interestingly, reduction in the scar volume, perfusion, and contractile function improvement was observed in the cell-transplanted regions. In addition to increased stroke volume, there was significantly increased ventricular wall thickening due to revascularization. It is important to mention that there was no control patient group in the study and results were compared to previously obtained data of post-CABG patients; hence, results should be viewed in scope of this important limitation of the PROMETHUS trial. However, these data favor MSC-based therapy in patients who require surgical intervention, as combining both approaches could lead to interesting outcomes.

The TAC-HTF Clinical Trial

Heldman and colleagues conducted TAC-HTF trial (ClinicalTrials.gov Identifier: NCT00768066), a phase I/II randomized, double-blind, placebo-controlled study (Heldman et al. 2014). Led by Joshua Hare at the University of Miami Miller School of Medicine, the trial was intended to study the safety and efficacy of trans-endocardial injection (TESI) of autologous human BM-derived mononuclear cells or MSCs in patients with chronic ischemic LV dysfunction and heart failure after MI. A total of 65 patients with confirmed ischemic cardiomyopathy and LVEF less than 50% were randomized to receive MSCs or mononuclear cells into ten different sites in the LV endocardially using an infusion catheter. No treatment-related SAEs were observed with statistical significance between the different arms of the study within 1 month, which was reassuring regarding the safety of the procedure. The study, however, recorded two unfortunate deaths due to cardiac adverse events, one patient each from the MSC- and placebo-treated groups at 239 and 115 days after respective treatment. A total of 16 patients were hospitalized, five patients each from the cell therapy groups and six patients from the placebo group. Six-MWT distance increased and infarct size decreased significantly within the group only in MSC-treated patients. The authors did not observe any significant change in LVEF and LV chamber volumes. The findings of this trial reassured the safety of TESI-based stem cell therapy in patients with chronic ischemic cardiomyopathy for larger future studies.

Mesenchymal Stromal Cell for IHD

Mathiasen and colleagues have reported a single center, randomized controlled study (ClinicalTrials.gov Identifier: NCT00644410). Led by Jens Kastrup at Rigs Hospitalet, Denmark, the trials were designed to study the effect of NOGA-guided direct intramyocardial injection of in vitro expanded MSCs on the development of new myocardium and blood vessels in patients with heart failure using saline-treated patient group as control (Mathiasen et al. 2012). A total of 60 patients (aged 30–80 years) with ischemic heart failure, LVEF <45%, and NYHA class II or III were randomized (2:1) to receive their respective treatment of cell therapy and placebo. Six-month follow-up revealed significant improvements in LVEF of 6.2% ($p = 0.0001$), stroke volume of 18.4 mL ($p = 0.0001$), and myocardial mass of 5.7 g (p ¼ 0.001) in cell therapy group vs placebo group (Mathiasen et al. 2015). The authors also observed reduced LVESV in MSC group, −7.6 (95% CI -11.8 to −3.4) mL ($p = 0.001$), which was increased in the placebo group, 5.4 (95% CI -0.4 to 11.2) mL ($p = 0.07$). However, no differences were observed in NYHA class, 6-MWT. The improvement was consistent and sustained even 1 year after treatment. Unfortunately, mortality was present in both intervention and control groups, but the mortality rate was less in MSC-treated group (35%) after 4 years of treatment than the control group (27.5%) (Mathiasen et al. 2020). A similar trend was seen in SAE as they were considerably lower in the treatment group, with fewer hospitalizations for angina. These data show the safety TESI and therapeutic benefits of autologous MSCs in patients with ischemic heart failure.

Intravenous Allogenic MSC Nonischemic Cardiomyopathy

Butler and colleagues have reported the results of a phase 2a, single-blind, placebo-controlled, randomized study that evaluated the safety and preliminary efficacy of intravenously administered allogenic BM-MSCs (Clinicaltrials.gov identifier: NCT02467387). A total of 22 patients with nonischemic heart failure were randomized to intravenously receive 1.5 million cells/kg ($n = 10$) or placebo control ($n = 12$) (Greene et al. 2017). The cells were expanded in vitro under chronic hypoxic conditions before transplantation to render them ischemia tolerant. The patients enrolled in this study had the specific characteristics of having chronic nonischemic cardiomyopathy with a LVEF 40% or less but without a prior history of MI and NYHA class II/III. After their respective treatment, the patients were followed up at regular of intervals of 30 days for the first 180 days, 9 months, and 15 months. There was no difference between the incidences of SAE in either cell therapy or control groups (Butler et al. 2017). There was one reported bruising at the site of intravenous infusion but no deaths or hospitalization in either group. Change in LVEF compared to the baseline was observed but insignificant. There was a finding of reductions in LV diastolic and systolic volumes. However, compared with placebo, cell therapy group showed an increased 6MWT distance (+36.47 m, 95% CI 5.98–66.97; $p = 0.02$). The authors attributed the functional improvement to the systemic anti-inflammatory and immunomodulatory effect of hypoxia-stimulated allogenic MSCs via the systemic release of bioactive molecules. Although the study was limited by the sample size and lacked a comparator group for hypoxia cultured cells using normoxia cultured cells, overall, intravenous administration of hypoxia preconditioned allogenic MSCs was safe and well tolerated intravenously and led to noticeable improvement in health status of the patients.

Conclusions and Future Perspective

Although the ongoing and already completed clinical trials diverge in the inclusion and exclusion criteria used for patient enrolment, the quality of the cell preparation, the number of cells injected, route of administration, number of injection sites for cell delivery, time of injection after ischemic episode, study endpoints and method of assessment, etc., the apparent divergence in all these parameters renders it challenging to reach to a consensus outcome in terms of MSC efficacy (Kallu et al. 2021). However, the current evidence stemming from these trials supports the safety and modest ability of MSCs to attenuate myocardial fibrosis after ischemia, besides neovascularization and tissue regeneration. A recently published systematic review and meta-analysis by Jeong et al. involved 950 patients from 14 randomized placebo-controlled trials that showed mechanical, regenerative, and clinical benefits of cell-based therapy using MSCs (Jeong et al. 2018). They observed 3.84% (95% CI: 2.3 ~ 5.35) increase in LEVF in MSC treatment group that was sustained until 24 months, while scar mass was reduced −1.13 (95% CI = −1.8 to −0.46). These observations comply with the outcome of a systematic review and meta-analysis published based on 23 preclinical studies in experimental animal models of AMI and

ischemic heart failure (Lalu et al. 2018). The observed therapeutic efficacy of stem cells may be improved further if certain requirements pertaining to the abovementioned parameters have been met and optimized.

One of these requirements, and possibly the primary one, is the quality of the cell preparation which is now being considered as responsible for modest outcome of the clinical trials (Haider 2018; Shahid et al. 2016). In this regard, the genetic constitution of the donor cells to ensure its acceptance by the recipient immune system is an important determinant to ensure that the transplanted cells survive and their derived tissue is sustained for a long time. Apparently, autologous MSCs seem an obvious choice as they inherently avoid any immunologic effects, but in the clinical perspective, this may not be true. For cardiovascular applications, patients who were candidates for cell therapy may not have the option of waiting for long enough that their autologous MSCs could be expanded in vitro to achieve them in sufficient number needed for transplantation. Similarly, aging and diseased autologous cells from the patient for engraftment in elderly patient may not be the best option in the clinic (Haider 2018). As the safety and efficacy of allogenic MSCs are comparable with autologous (Jansen of Lorkeers et al. 2015) and even better in some cases than the autologous cells, the use of allogenic MSCs has logistic superiority. Please refer to ► Chap. 1, "Human Mesenchymal Stem Cells: The Art to Use Them in the Treatment of Previously Untreatable," in this book for further discussion where a case for allogenic MSCs has been discussed in depth.

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Preclinical Research of Mesenchymal Stem Cell-Based Therapy for Ocular Diseases

11

E. Zakirova, A. M. Aimaletdinov, A. G. Malanyeva, C. S. Rutland, and A. A. Rizvanov

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Abstract

Mesenchymal stem cells (MSC) are specific cell types that enable tissue renewal within the body and are activated during the regeneration processes in response to injury. These cells are present in all human and animal tissues. In medical and biological research, MSCs are isolated and purified from the donor organism and cultured in vitro before use in the treatment of a variety of diseases and conditions associated with tissue damage and cell loss, including ocular lesions. This approach is called cell-based therapy or regenerative medicine. Despite the many existing therapeutic strategies in the area of cell therapy in relation to curing ocular diseases in recent years, advances and new regenerative therapy

E. Zakirova · A. M. Aimaletdinov · A. G. Malanyeva · A. A. Rizvanov (✉)
Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

C. S. Rutland
Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Science, University of Nottingham, Nottingham, UK
e-mail: catrin.rutland@nottingham.ac.uk

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methods have also been developed and consolidated, giving us a new perspective. Therefore, it is necessary to implement standardization and comparison of cell therapy results. Experimentation using animal models has played a central role in biomedical research. The safety and efficacy of new drugs are usually tested in animal models of human diseases prior to entering human clinical trials. Nevertheless, the pathophysiological mechanisms of eye diseases are complex and multifactorial; hence it is crucial that experimental animal models with clinical relevance provide adequate information and sufficiently replicate the eye diseases being assessed and demonstrate the effects of MSC therapy.

Keywords

Experimental · Eye · Humans · MSCs · Ocular · Regenerative

Abbreviations

ADMSC	Adipose-derived mesenchymal stem cells
AMD	Age-related macular degeneration
BDNF	Brain-derived neurotrophic factor
BMSC	Bone marrow stromal cells
CNV	Choroidal neovascularization
DR	Diabetic retinopathy
ES	Embryonic stem cells
GCL	Ganglion cell layer
GFP	Green fluorescent protein
PVPC	Multipotent perivascular progenitor cells derived from human
hESC	Embryonic stem cells
hRPC	Human retinal progenitor cells
LEC	Lens epithelial cells
LHON	Leber's hereditary optic neuropathy
MSC	Mesenchymal stem cells
NF1	Neurofibromatosis type 1
POAG	Primary open-angle glaucoma
RCS	Retinal dystrophy of Royal College of Surgeons rats
RGC	Retinal ganglion cells
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelial cells
SC	Stem cells
UMSC	Umbilical cord mesenchymal stem cells

Introduction

Dysfunction of the visual system can significantly reduce the quality of human life, as the brain receives more than 80% of the incoming information via the eyes. According to the World Health Organization (WHO), in 2020, there were more than

1.3 billion people with visual impairments; 217 million people within this group had poor vision, and 36 million were blind. The leading causes of visual impairment are uncorrected refractive errors, cataracts, age-related macular degeneration, glaucoma, diabetic retinopathy, and corneal opacity (Lancet Global Health 2019).

Despite the availability of various medications and therapies available for use in ophthalmic practice presently, not all ocular diseases are treatable. Therefore, stem cell (SC) therapy has recently become more widespread. Many publications indicate its effectiveness in treating intractable eye diseases for both humans and animals (Zakirova et al. 2015, 2019).

The role of experimental animal models for obtaining information about the effectiveness and safety of cell therapy, including SC therapy, has significantly increased. Most studies are currently conducted on rodents, and on large animals, such as rabbits, dogs, pigs, sheep, goats, and non-human primates (Harding et al. 2013).

There is presently a wide range of potential SC-based drugs that can be used for medical purposes. Currently, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) somatic stem cells, as well as differentiated cells derived from SC, mesenchymal stem cells (MSCs) from adipose tissue (ADMSCs), MSCs from bone marrow (BM-MSCs), neural stem cells, endogenous retinal stem cells, and stem cells derived from ciliary, retinal pigment epithelium, and umbilical cords (UMSCs) are used for therapy (Harding et al. 2013).

Currently, preclinical animal models are used to develop methods for treating eye surface diseases, glaucoma, retinal diseases, diabetic retinopathy, and age-related macular degeneration. Many experimental animal models imitate eye diseases, but none of them reflect the incredible complexity that the human disease possesses. Therefore, to replicate or emulate different aspects of human pathology, several experimental models of the same disease are usually required. Some of the important and commonly used experimental animal models used for testing cellular technologies in ophthalmology are summarized in Table 1.

Models of Corneal Diseases

The cornea is a piece of transparent tissue in the anterior aspect of the eye. It is a protective physical and biological barrier against the external environment and provides a refractive force for the concentration of light on the retina (Lewis et al. 2010).

The cornea comprises three main cell types: stratified surface epithelium, stromal keratocytes, and the innermost single-layered endothelial cells, which are neuroepithelial in nature (Sagizade et al. 2017). Surface epithelial cells are essential for corneal transparency and visual acuity. Corneal epithelial cells are continuously enhanced by a population of SC or progenitor cells, which are located in the corneal limb. However, severe corneal damage caused by chemical or mechanical exposure, and immune or hereditary diseases, can lead to corneal inflammation, ulceration, neovascularization, and deficiency of limbal SC. All of these situations can result in loss of vision.

Table 1 Animal models used for testing cellular technologies in ophthalmology

Eye tissue	Animal	Model	Cell type	References
Cornea	Mouse	The transgenic model. Lumican UMSC knockout mice.	UMSC	Liu et al. (2010)
		Transgenic mice model with mucopolysaccharidosis type VII	UMSC	Coulson-Thomas et al. (2013)
		Mechanical damage	BMSC	Shukla et al. (2019)
		Laser induction of damage	ADMSC	Zeppieri et al. (2017)
		Thermal damage	BMSC	Lan et al. (2012)
	Rat	Chemical burns	BMSC	Ma et al. (2006)
		Chemical burn with alkali	MSC	Faruk et al. (2017)
			BMSC	Li et al. (2015)
			MSC	Yao et al. (2012)
		Chemical damage by 100% ethanol	MSC	Oh et al. 2008
			BMSC, corneal epithelial cells	Oh et al. (2009)
		Surgical injury	ADMSC c PAX6	Joe and Gregory-Evans (2010)
		The dry eye syndrome caused by benzalkonium chloride	MSC	Beyazyıldız et al. (2014)
	Rabbit	Chemical alkali burn	MSC	Ye et al. (2006)
			BMSC	Gu et al. (2009)
		Surgical injury	ADMSC	Galindo et al. (2017)
			BMSC	Then et al. (2017)
		Model of bullous keratopathy	Primary corneal endothelial cells	Peh et al. (2017)
	Dog	Spontaneous model of dry keratoconjunctivitis	MSC	Sgrignoli et al. (2019)
		Spontaneous corneal wounds and ulcers	MSC	Falcão et al. (2019)
Lens	Rat	Induced by sodium selenite-	Wharton jelly MSCs	Maleki (2015)
		Model of diabetes 2 type	ADMSC	Yu et al. (2019)
	Rabbit, Macaque	Surgical method	LEC	Haotian et al. (2016)
Retina		Laser induction	BMSC, RPE	Hou et al. (2010)
	Rat	Diabetes model induced by streptozotocin	hESC-PVPC BMSC	Kim et al. (2016)

(continued)

Table 1 (continued)

Eye tissue	Animal	Model	Cell type	References
	Rat	RCS rats with dystrophy	hRPC hRPC/hBMSC RPE RPESC-RPE	Semo et al. (2016), Zhao et al. (2017), McGill et al. (2017), Davis et al. (2017)
	Rat	Model of retinal degeneration by sodium iodate injection	hESC	Park et al. (2011)
	Mouse	Transgenic model, Rd1	ESC и IPSC Precursors of rods	Assawachanaont et al. (2014), Barnea-Cramer et al. (2016), Singh et al. (2013)
	Rat	Optic nerve injury	MSC MSC from cord blood	Mesentier-Louro et al. (2014), Zwart et al. (2009)
	Rat	NMDA model	ESC	Aoki et al. (2008), Divya et al. (2017)
	Cat	NMDA model	MSC from Muller's glia	Becker et al. (2016)

Corneal transplantation is currently a reasonably effective method for managing corneal damage. However, the main problem with this treatment is the rejection of the transplants following this therapeutic technique. The use of allogeneic or autologous limbal cell transplantation is also limited due to the significant risk of systemic immunosuppression. Autologous transplantation can also lead to damage in the contralateral normal eye. Therefore, the search for a safe and adequate source of cells to create a bioengineered corneal epithelium is still ongoing, and one which is under active research at the present.

Numerous experimental studies have attempted to use mesenchymal stem cells (MSCs) as a bioengineered corneal epithelium. Research has also confirmed that epithelial-like corneal cells can be formed from mesenchymal and embryonic stem cells (ESCs) extracted from bone marrow. The numerous potential advantages of adipose tissue-derived MSCs (ADMSC), including easy availability, low immunogenicity, high pluripotency, lack of ethical controversy, and reduced risk of infection, have made them a particularly attractive cell source for bioengineered corneal epithelium (Sun et al. 2018).

MSCs can treat both congenital corneal diseases and various types of injuries and damages (Joe and Gregory-Evans 2010). Additionally, there are many animal models designed to reproduce inherited diseases and injuries affecting the cornea. Therefore, the therapeutic efficacy of MSCs has been tested on various animals (mice, rats, rabbits, dogs, and primates).

In addition to different species, different model types have been used, including transgenic animals, experimental animals with natural spontaneous corneal diseases, and induced models.

Liu and colleagues used knockout mice as model animals for the Lumican gene (Liu et al. 2010). The mice received UMMC transplantations to facilitate studying their potential use as a therapy for congenital corneal diseases caused by genetic mutations. As a result, it was found that transplantation into the UMMC corneal stroma increases the thickness of the stroma and reduces corneal opacity. The turbidity of the corneal stroma was determined by measuring the total pixel intensity of light scattering through a three-dimensional volume, while the thickness of the corneal stroma was calculated by measuring the axial distance from the anterior to posterior stroma (Liu et al. 2010).

Coulson-Thomas and Caterson used genetically modified MPS VII mice with type VII mucopolysaccharidosis as a model to study the therapeutic properties of mesenchymal stem cells (Coulson-Thomas and Caterson 2013). Their study was aimed to establish cell therapy effectiveness in mucopolysaraidosis VII (Sly syndrome). The researchers used UMSCs, which were transplanted into the corneal stroma. A significant number of the UMSCs delivered to the cornea survived the transplant process and remained in the cornea throughout the treatment period. The data obtained showed that human UMSCS transplanted into the murine cornea with MPS VII prevented the accumulation of glycosaminoglycans in the corneal stroma and keratocytes. There was also a lack of corneal opacity, which showed that UMSCs could prevent the disease progression (Coulson-Thomas et al. 2013).

Shukla and Mittal (2019) used a mechanical approach to damage the cornea. The corneal epithelium and anterior stroma, which account for approximately one-third of the total thickness of the cornea, were mechanically removed using an Algerbrush brush. The study was conducted to determine the effectiveness of BMSCs on damaged cornea and conjunctiva within the eye. The cells were injected subconjunctivally, intravenously, and intraperitoneally for an hour after the injury had been induced. Studies have shown that subconjunctival or intravenous administration of BMSCs has higher therapeutic efficacy compared to local or intraperitoneal administration following corneal damage. The authors noted a decrease in corneal opacity, tissue fibrosis, and the expression of pro-inflammatory cytokines (Shukla et al. 2019).

In a laser-induced model of corneal damage in mice, ADMSC administration had significantly smaller defects of corneal epithelial (Zeppieri et al. 2017). In a thermal moxibustion murine model of corneal damage, the ability of MSCs to penetrate into damaged tissue and promote repair of corneal was investigated (Lan et al. 2012). BMSCs were administered intravenously following injury, and the outcomes were evaluated using epifluorescence microscopy whereas the epithelial regeneration was evaluated via corneal fluorescein staining. Studies have shown that the systemic administration of MSCs affected the damaged cornea and showed their long-term survival. The researchers also noted that after the cornea had been damaged, the introduction of BMSCs resulted in a significant and rapid regeneration of the corneal epithelium (Lan et al. 2012).

The effectiveness of human BMSCs in treating chemical burns in the corneal epithelium of rats has also been investigated. BMSCs were cultured on the human amniotic membrane, and 7 days after a chemical burn had been administered, amniotic membranes with BMSCs were transplanted onto rodent corneas. The

anatomical effects and vision of these rodents were measured once a week with a slit lamp and optokinetic head tracking response, respectively. The resulting data showed that BMSCs successfully repaired damaged corneal surfaces in rats (Ma et al. 2006).

Faruk et al. (2017) induced corneal damage using an alkali solution in rats. Studies have shown that systemic injection of autologous MSCs leads to regeneration of the corneal epithelium and inhibits neovascularization in the induced chemical burn of the cornea. The authors showed that MSCs had a therapeutic effect and inhibited early inflammatory responses in local corneal cells (Faruk et al. 2017). This method of corneal damage has also been used in rats. A model of corneal burn, using an alkali, was created by placing a 3 mm diameter piece of NaOH-soaked filter paper onto the right eyes of rats. Immediately after the injury, and 3 days later, the rats were given a subconjunctival injection containing a 2×10^6 MSCs suspension. Studies have shown that subconjunctival injection of MSCs reduces inflammation of the locally burned cornea by inhibiting the infiltration of inflammatory cells and the production of pro-inflammatory cytokines (Yao et al. 2012).

The therapeutic effects of MSCs have also been tested in a model where 100% ethanol was applied to the eye's surface. Afterward, a reservoir with a hollow tube pre-filled with MSCs and culture medium was placed on the cornea and left in situ for 2 h. The reservoir was then removed and the eyes sutured. In this study, the authors demonstrated that MSCs reduced corneal inflammation and neovascularization and this was associated with IL-10 and TGF-beta1, IL-6, and TSP-1 upregulation, and IL-2, IFN-gamma, and MMP-2 downregulation, as well as decreased tissue infiltration by CD4 + cells (Oh et al. 2008). The same researchers also applied the same rat model of 100% ethanol chemical damage where BMSCs and human corneal epithelial cells were directly injected into the cornea. This technique resulted in upregulated expression of IL-6, VEGF, TGFbeta1, MMP-2, and thrombospondin-1, and suppression of MMP-9 secretion by the damaged epithelial cells (Oh et al. 2009).

One study investigated surgical corneal damage with ADMSC with PAX6 therapy. The research revealed that PAX6 induces differentiation of ADMSCs into epithelial-like corneal cells in vitro. ADMSCS reprogrammed with PAX6 also repaired damaged corneal surfaces in vivo (Joe and Gregory-Evans 2010).

The therapeutic efficacy of systemic administration of BMSCs after applying an alkaline burn to the experimental rats was investigated. Efficacy was assessed by corneal reepithelization, corneal opacification, and neovascularization. In the publication, the authors concluded that systemically transplanted BMSCs can take root in the damaged cornea, promoting wound healing through differentiation, proliferation, and synergy with hematopoietic stem cells (Li et al. 2014).

The therapeutic efficacy of topically applied MSCs for dry eye syndrome has been extensively reported (Hirayama 2018; Villatoro et al. 2017). The effectiveness of topical application of MSCs was assessed to treat dry eye syndrome (keratoconjunctivitis sicca; KCS) caused by benzalkonium chloride (BAC) in an experimental rat model. Eye drops containing ADMSC were applied topically every day for a week. The effectiveness of the treatment was evaluated using the Schirmer test, evaluation of the time of destruction, assessment of the eye surface, the index of

corneal inflammation, and histological and electron microscopic analysis. Studies have shown that topical application of ADMSC can be a safe and effective treatment for dry eye syndrome (Beyazyıldız et al. 2014).

Mesenchymal stem cells (MSCs) are also known to promote the engraftment of cell and organ transplants due to their immunotherapeutic and immunomodulatory characteristics (Beeken et al. 2021). Indeed subconjunctival injection of MSCs to rats was effective in increasing the survival rate of corneal allografts. This effect was due to the inhibition of inflammatory and immune responses (Jia et al. 2018).

The possibility of using MSC systemic administration to accelerate the healing of corneal wounds after the alkaline burn of rabbits has been investigated. The clinical outcomes were evaluated using corneal reepithelization, corneal opacification, neovascularization, and immunohistochemical studies. The experiments conducted showed that systemically transplanted MSCs could engraft the damaged cornea, stimulating wound healing by differentiation, proliferation, and synergy with hematopoietic stem cells (Ye et al. 2006).

Gu and Xing (2009) studied the efficacy of MSCs in rabbits concerning whether BMSCs can differentiate into corneal epithelial cells. Corneal damage in rabbits was caused by contact with 1 N NaOH-soaked filter paper for 30 s. Twenty-eight days after corneal injury, fibrin gels were transplanted onto the rabbit cornea. The corneas of the rabbits were observed each day for follow-up with a slit-lamp microscope system (SL-1600; Nidek Co., Ltd., Aichi, Japan) to assess reepithelization, neovascularization, and transparency. The data showed that following BMSC transplantation, the damaged surface of the rabbit cornea was successfully reconstructed, and some of the transplanted cells directly participated in the healing process of the damaged corneal epithelium (Gu et al. 2009).

The effects of human ADMSCs on surgically damaged cornea have also been investigated. Studies have shown that ADMSCs transplanted to the surface of the eye migrated to the inflamed tissues, reduced inflammation, restrained the development of neovascularization and corneal opacification, and partially restored the phenotypes of the limbal and corneal epithelium (Galindo et al. 2017). Molecular profiling revealed partial restoration of corneal epithelial cell markers CK3 and E-Cadherin and the limbal epithelial cell markers CK15 and p63, that was lost during experimental corneal failure. The same research group has reported that subconjunctival injection of MSCs is less invasive and allows high-dose administration of MSCs besides being more efficacious (Galindo et al. 2021).

Evaluation of autologous BMSCs for the treatment of corneal stroma defects in rabbits showed interesting preliminary results. Animals were subjected to deep lamellar corneal dissections, and clinical outcomes were assessed via corneal reepithelization, corneal opacity, corneal thickness, and histology. Studies have shown that the use of BMSCs did not achieve complete transparency in the cornea. It has been suggested that remodeling the corneal stroma to fully restore the original optical qualities requires a longer time ranging from several months to several years. The authors noted that locally transplanted BMSCs could be a valuable source of corneal stroma regeneration (Then et al. 2017).

The beneficial therapeutic effect of isolated primary human corneal endothelial cell transplantation on a preclinical model of bullous keratopathy in rabbits has shown promising results. Primary corneal endothelial cell transplantation gradually reduces the thickness of the cornea during the first 2 weeks. It completely restores it to a thickness of approximately 400 microns by the third week of transplantation. In contrast, the cornea of control rabbits remained significantly thicker, more than 1000 microns ($p < 0.05$) throughout the study. This study data showed that the use of primary human corneal endothelial cells opens up excellent prospects in the clinic (Peh et al. 2017).

In addition to experimental animal models, it has been highlighted that veterinary patients, such as dogs, are increasingly recognized as critical translational models of human diseases. For example, the etiopathogenesis of canine diseases is similar to that of humans (Brown 2016). Therefore studies of keratoconjunctivitis in dogs can help to develop therapeutic approaches which may also benefit people. Researchers have evaluated the intralacrimal transplantation of allogeneic MSCs in dogs with mild, moderate, and severe dry conjunctivitis. The data retrieved showed that allogeneic transplantation of MSCs on dogs was safe as no side-effects were observed with allogenic MSCs. The authors also showed improvements in the condition of the treated eyes. Experiments have shown that the use of MSCs is effective and safe for experimental dogs and, therefore, this type of treatment may be used in clinics after additional tests (Bittencourt et al. 2016).

In another canine study, this time in corneal wounds, the therapeutic efficacy of MSCs was tested in dogs. Previously, the experimental animals had been diagnosed with deep corneal ulcers, and for the duration of the experiment, the animals did not receive any immunomodulatory drugs. The researchers noted that all dog owners had signed written consent before this experimental procedure was started. Moreover, all owners were fully informed that the safety, complications, and effectiveness of cell implantation in corneal ulcers were unknown and not very well-established. The effectiveness of the treatment was evaluated using ophthalmological examination on three occasions, once prior to MSC therapy, then on days seven and 14 following their respective treatment. The tests included criteria such as clinical signs and the size and depth of the ulcers. MSCs were administered sub-conjunctively at a dose of three million cells. Studies have shown that the effectiveness of treatment was 84.6%, that is, the healing was observed in 22 of the 26 experimental dogs (Falcão et al. 2019).

Keratoconjunctivitis, or dry eye syndrome, is mainly an immuno-mediated degenerative disease directly affecting patients' vision and quality of life; it is one of the leading causes of ocular diseases in both dogs and humans. Dogs are excellent animal models for facilitating the understanding of this disease. One trial used 22 animals that were already unwell to study the effectiveness of MSCs. MSCs were administered locally and the treatment effectiveness was evaluated based on clinical signs, ophthalmological tests, histology, and immunohistochemistry. The work showed that the introduction of MSCs improved the condition of the cornea in experimental dogs, and relapses were only observed in seven of the 22 treated dogs (Sgrignoli et al. 2019).

Models of Lens Diseases

The structure of the human lens is reflected in Fig. 1.

A cataract is a pathological condition associated with the opacity of the eye lens which causes varying degrees of visual impairment up to complete loss of vision. A cataract is one of the problems that affect the clarity of vision and can be caused by a number of factors, including aging, eye injuries, inflammation, and other circumstances. According to the latest estimate, cataract occurrence is responsible for 51% of the world's blindness, affecting around 20 million people. An effective way to treat a cataract is surgery; the natural lens of the eye is removed and replaced with an artificial lens (Snellingen et al. 2002).

Maleki (2015) induced lens damage in rats and rabbits via administration of sodium selenite. They conducted research to identify the therapeutic properties of SCs derived from Wharton's jelly by introducing SCs into the lens via surgical manipulation. The effectiveness of this treatment was evaluated by determining the expression of βB1 and βB3-crystallin genes and by evaluating the lens' morphological ultrastructure by electron microscopy. Studies have shown that the SCs obtained from Wharton's jelly, differentiated into the lens fibers and then restored lens structure within the capsule. The authors suggested that this method of cataract treatment could be applied in clinical practice following additional safety parameter studies (Maleki 2015).

To evaluate the complications associated with the development of type 2 diabetes pathologies, the effectiveness of ADMSC was investigated. This work illustrated the positive effects on various body systems in the experimental rats, including the lens of the eye. In the group of animals that received ADMSC intravenously, lens opacification was not observed, results which contrasted to those observed in the control group (Yu et al. 2019).

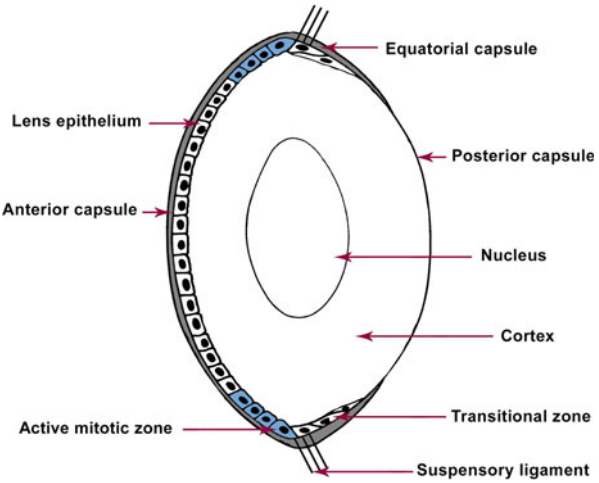

Fig. 1 Diagram showing the structure of the human lens

A new surgical method for the treatment of cataracts, which consisted of preserving endogenous lens progenitor cells (LEC), was developed as it was thought that the cells might provide functional regeneration of the lens in rabbit and macaque models. Although this method is conceptually different from modern practice, since it preserves the endogenous LEC and their natural environment as much as possible, it also restores the functionality of the lens (Haotian et al. 2016).

Retinal Models

Retinal diseases are one of the leading causes of blindness in the modern world. Previous research has shown that many retinal diseases observed in animals are similar to those seen in people. This work has primarily led to an improved understanding of the pathogenesis of retinal diseases and has also provided the means to test possible treatments, including cell therapy.

In the diseases of the external retina, such as age-related macular degeneration (AMD) and diabetic retinopathy (DR), two of the most common causes of blindness in the developed countries, the main body of in vivo models are those showing abnormal retinal angiogenesis or damage to the retinal pigment epithelium (RPE) (Liu et al. 2017).

One model with induced choroid neovascularization (CNV) caused by a laser was used to study the therapeutic effect of bone marrow-derived cells. The intravenously administered MSCs penetrated the damaged area and differentiated into several cell types and participated in the development of neovascularization without stagnation in other organs, thereby suppressing the growth of choroid neovascularization (Hou et al. 2010). Li and colleagues also used laser photocoagulation to induce CNV to investigate whether subretinal transplantation of RPE expressing Fbln5 could suppress CNV in vivo. One week after laser-induced CNV, RPE cells were transplanted into the subretinal space, with the pZlen-Fbln5-IRES-GFP vector placed in the right eye and the pZlen-IRES-GFP vector in the left eye. CNV was then evaluated using fundus photography, fundus fluorescence angiography, and hematoxylin and eosin staining. CNV appeared 1 week after photocoagulation and reached a peak of activity after 3 weeks. The transplanted RPE cells survived for at least 4 weeks and migrated to the retina. Subretinal transplantation of RPE cells, with Fbln5 expression, resulted in a significant reduction in the total lesion area. The researchers concluded that subretinal transplantation of RPE cells with Fbln5 expression inhibited laser-induced CNV in rats and therefore, represented a promising therapy for this condition (Li et al. 2015).

Neovascularization has been reproduced in a diabetes model induced by the administration of STZ in rodents. It was hypothesized that multipotent perivascular progenitor cells derived from human embryonic stem cells (hESC-PVPC) would improve the damaged retinal vasculature. Indeed a single intravitreal injection into Brown Norway diabetic rats caused the localization of cells in distinct perivascular areas of the retinal vasculature and stabilized the rupture of the hemato-retinal barrier. This study shows the therapeutic potential of hESC-PVPCs in diabetic

retinopathy by imitating the role of pericytes in vascular stabilization. The researchers highlighted that this study represents a simple method for creating perivascular progenitor cells from human embryonic stem cells. These cells have common functional characteristics with pericytes, which are irreparably lost at the onset of diabetic retinopathy. Animal studies have shown that replenishing damaged pericytes with perivascular progenitor cells can restore the integrity of retinal vessels and prevent loss of vitreous fluid. These data provide promising and compelling evidence that perivascular progenitor cells could be used as a novel therapeutic agent to treat patients with diabetic retinopathy (Kim et al. 2016).

The effectiveness of BMSCs in diabetic retinopathy was studied in a streptozotocin (STZ) model in male albino Wistar rats. After 3 months of induced diabetes, the right eye was intravitreally injected with green protein-labeled guidelines and the left eye was injected with a balanced saline solution. The introduction of BMSCs increased retinal gliosis in the diabetic group compared to the control group of animals. Immunofluorescence analysis revealed that BMSCs mainly integrated into the inner retina. Overall, this study showed that intravitreal administration of BMSCs improved visual function (Çerman et al. 2016). Attention is currently being paid to models with photoreceptor damage from retinitis pigmentosa and various hereditary disorders of central vision. It should be noted that these animal models are a target for the transplantation of early photoreceptors or RPE cells because RPE cells are responsible for protecting the function of photoreceptors.

The efficacy and safety of human retinal progenitor cells (hRPCs) in experimental RCS rats with dystrophy have also been researched. The hRPCs have been tested to maintain visual function and evaluation was undertaken using optokinetic head tracking and retinal structure studies. The safety of the NPHR was evaluated by subretinal cell transplantation into rats and wild-type mice of the bodily lower-III stages with analysis at 3, 6, and 9 months after transplantation, respectively. Studies have shown that the optimal dose of hRPCs for preserving visual function and retinal structure in dystrophic rats was between 50,000 and 100,000 cells. The human retinal progenitor cells integrated and survived in the retinas of dystrophic and wild-type rats up to 6 months after transplantation. No signs of tumors were detected. Therefore human retinal progenitor cells appear to be safe and effective in this preclinical model. It has been shown that they can be used in the early stages of retinal degeneration and the areas of intact retina without the risk of adverse effects on visual function (Semo et al. 2016).

Qu, Linghui et al. (2017) studied the effects of both hRPCs and BMSCs in a different disorder, retinitis pigmentosa (RP). The researchers have noted that both hRPCs and human BMSCs (hBMSCs) are widely and practically used for transplantation. In this study, the possibility of using combined transplantation of the above cells was tested. The researchers transplanted hRPCs and hBMSCs into the subretinal space of RCS rats. Studies have shown that combined hRPCs/hBMSCs transplants supported electroretinogram results much better than single transplants. The thickness of the outer nuclear layer also showed the best results during combined transplantation. It was also recorded that the cells in the combination treatment migrated better than single transplantation. Photoreceptor differentiation of cells in

the retina rats treated with combined cell transplantation also showed a higher ratio than those treated with a single type cell transplant. Finally, microglial activation and Muller cell gliosis were more effectively suppressed following a combined transplantation protocol, indicating better immunomodulatory and anti-glial effects. The researchers stated that the combination of hRPC and hBMSC transplantation was a more effective strategy for treating SC-based retinal degenerative diseases (Qu et al. 2017).

Substitution therapy in degenerative diseases such as age-related macular degeneration has also been investigated. A modified trans-scleral injection method has been developed that uses specific angles and needle depth to successfully and consistently deliver retinal pigment epithelial cells into the rat's subretinal space and prevent excessive retinal damage. Efficacy was demonstrated in RCS rats sustaining for 2 months (Zhao et al. 2017).

The efficacy of RPE cells obtained under xenon-free conditions from clinical and xenon-free human embryonic stem cells (OpRegen) after transplantation into the subretinal space of the RCS rats has also been characterized. The rats received a single subretinal injection of xeno-free RPE cells, whereas a physiological saline solution and non-operated eyes served as a control. Optomotor behavior tracking was used to evaluate functional effectiveness and recovery of photoreceptors, and survival of the transplanted cells was assessed using histology and immunohistochemistry. The subsequent studies showed that the outer nuclear layer (ONL) was significantly thicker in the eyes treated with the cells than in the controls. Transplanted RPE cells were identified in the subretinal space, and integration into the RPE monolayer was also revealed. It was proven that the OpRegen RPE cells survived and restored visual function and preserved rod and cone photoreceptors for an extended time period (McGill et al. 2017).

During subretinal transplantation of RPE-derived stem cells (RPESCs) and RPE cells (RPESCs-RPE), the preserved vision was observed in a model of RPE cell dysfunction in RCS rats. The researchers noted that the stage of differentiation that the RPESCs-RPE had acquired before transplantation significantly affected the effectiveness of vision restoration. While the cells at all tested stages of differentiation protected photoreceptor layer morphology, the intermediate stage of RPESCs-RPE differentiation, obtained after 4 weeks of culture, was more consistent in protecting vision than the subsequent generation that differentiated during culture weeks 2 or 8 (Davis et al. 2017).

Park and colleagues used an experimental rat model of retinal degeneration developed by an intravenous injection of sodium iodate. Human embryonic stem cells (hESCs) as a therapeutic agent for treating degenerative retinal diseases and proposed to differentiate the cells into retinal pigment epithelium (RPE) using particular culture conditions. The putative RPE cells (10^5 cells/5 μl) were transplanted into the subretinal space. The animals were killed at either 1, 2, or 3 weeks after transplantation for immunohistochemistry to check transplanted cell survival and their fate. The putative RPE cells derived from hESCs had the morphological characteristics of human RPE cells. The implanted RPE cells survived in the subretinal space for up to 4 weeks post-transplantation, and the expression of RPE

markers was confirmed via immunohistochemistry. Thus, the researchers showed the potential of using hESC-derived RPE cells for cellular therapy of retinal degenerative disease (Park et al. 2011).

In another model of retinal degeneration, Rd1 mice were used to replicate rapid progressive retinitis pigmentosa with end-stage retinal degeneration. Development of a structured outer nuclear layer with internal and external segments during the transplantation and integration of three-dimensional retinal tissue derived from mouse embryonic stem cells or induced pluripotent stem cells were investigated. The resulting retinal sheets were transplanted into the subretinal space of mice aged 6–8 weeks and sacrificed from 2 weeks to 6 months after transplantation. The transplanted retinal sheets obtained from both mESCs and iPSCs survived and matured in the highly degenerated retinas of the experimental animals. This study is a "proof-of-concept" for retinal transplantation in late retinal degenerative diseases (Assawachananont et al. 2014).

The therapeutic potential of photoreceptor precursors derived from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have been studied to confirm their potential in retinal degeneration. ESCs and iPSCs were cultured in four stages under certain conditions, resulting in a somewhat homogeneous population of photoreceptor-like progenitors. After transplantation into the experimental mice model of end-stage retinal degeneration, these cells differentiated into photoreceptors and formed a cell layer associated with the host retinal neurons. Visual function in the animals of the experimental group was partially restored, as evidenced by visual behavioral tests. In addition, the degree of functional improvement was positively correlated with the number of implanted cells. The data obtained confirmed the potential of ESCs and iPSCs for photoreceptor replacement therapy aimed at photoreceptor regeneration in retinal diseases (Barnea-Cramer et al. 2016).

An experimental mouse model of severe human retinitis pigmentosa at a stage of loss native cells showed that transplanted rod precursors could transform an anatomically distinct and appropriately polarized outer nuclear layer. The three-layer organization was returned to rd1 mice that had only two retinal layers before treatment. The transplanted progenitors could integrate and develop in the degenerated retina and transformed into mature rods with light-sensitive outer segments connected to neurons. The visual function in these experimental animals was also restored after cell transplantation. Studies have shown that cell therapy can restore the light-sensitive cell layer de novo and, consequently, restore the structurally damaged visual contour (Singh 2013).

Diseases of the internal retina, such as glaucoma and various forms of optical neuropathy (ischemic, traumatic, and compression), have common characteristics of retinal ganglion cell loss (RGC). Experimental animal models that focus directly on RGC damage do not necessarily reproduce the pathogenesis of individual conditions; however, they are widely used because of their ability to demonstrate dramatic changes in RGC function in many different animal types. Damage to the optic nerve is one of the frequently used preclinical methods to reproduce the loss of RGCs. This model was used to study RGC stimulation as a way to regenerate

axons along the entire length of the visual pathway and further in the lateral geniculate nucleus, the upper mound, and other visual centers. Notably, regeneration partially restored the optomotor response, demonstrating the possibility of restoring the central vision circuit after optic nerve damage in adult mammals (de Lima et al. 2012).

The therapeutic potential of MSCs injected into the vitreous body, also on a model of optic nerve damage, has been demonstrated. Adult (three to five-month old) Lister hooded rats underwent unilateral optic nerve crushing followed by an injection of MSCs into the vitreous. Before injection, MSCs were labeled with a fluorescent dye or superparamagnetic iron oxide nanoparticles, which enabled cell tracking and their post-engraftment fate determination in vivo using magnetic resonance imaging. Retinal ganglion cell survival was evaluated at 16 and 28 days after the injury. The researchers noted that the transplanted MSCs remained in the vitreous body and were detected in the eye for several weeks. The MSCs-based therapy's success was further explained by an increase in FGF-2 expression in the retinal ganglion cell layer, and the concomitant expression of interleukin-1β. Thus, studies have shown that MSCs protect RGCs and stimulate axon regeneration following optic nerve damage (Mesentier-Louro et al. 2014).

Evaluation of human UMSCS to protect and promote the regeneration of axotomized neurons in the rat optical system "UMSK" has also been undertaken. The cells survived well up to 2 weeks post-transplantation; however, they did not significantly migrate significantly or differentiate. In the presence of UMMC grafts, it was found that the axonal processes of the host were present at the site of the lesion, and there was a stimulation of the population of endogenous neural progenitors. Four weeks after the transplant, UMMC was shown to have a neuroprotective effect, protecting a significant percentage of axotomized retinal ganglion cells. Further experiments showed that UMSCS could also promote the re-growth of axotomized RGCs (Zwart et al. 2009).

Model Damage to the *N*-methyl-d-aspartate Receptor (NMDA)

Glutamate excitotoxicity is a critical component of selective neuronal cell death in ischemic retinopathy and glaucoma. This process is associated with excessive stimulation of NMDA receptors in retinal ganglion cells, which leads to an intracellular influx of calcium ions, and this, in turn, leads to the activation of the apoptotic cascade. Experimental animal models using glutamate excitotoxicity focus on NMDA receptor stimulation have been developed and optimized (Casson 2006). This was used to study a constructed eye-like structure derived from ESCs consisting of retinal neural clone cells, RPEs, and lens cells to determine whether they could differentiate into retinal ganglion cells (RGC) during retinal transplantation into the vitreous body of a damaged adult mouse (Aoki et al. 2008). Prior to this, it was shown that the cells of these eye-like structures could integrate into the developing visual bubble of chickens. ESCs were induced to differentiate into

eye-like structures in vitro for 6 or 11 days. NMDA was then injected into the eyes of the mouse recipients to damage the RGC before transplantation. Cell material for transplantation was extracted from eye-like structures and transplanted into the vitreous body of both the damaged and control eyes. During the follow-up, the eyes were analyzed both qualitatively and quantitatively by immunohistochemistry 10 days or 8 weeks after transplantation. Studies showed that cells from eye-like structures derived from ESCs had integrated into the RGC layer and differentiated into neurons when transplanted into control (non-NMDA-treated) eyes; however, they rarely expressed RGC markers. Once transplanted into NMDA-treated eyes, the cells spread across the surface of the retina and covered a relatively large area of the host's RGC layer, which was damaged by NMDA. Eye cells derived from ECU often differentiated into cells expressing RGC -specific markers and formed a new layer of RGC. In addition, it was observed that a small number of these cells, originating from the ESC, expanded the axon-like processes in the direction of the optic disc. However, visually induced responses could not be recorded from the visual cortex (Aoki et al. 2008).

Transplantation of neural progenitors, derived from ECU (Es-NP) into *N*-methyl-D-aspartate (NMDA)-injected mouse models, ablated RGA in mouse models, and preclinical glaucoma (dBA/2D) models with consistently higher intraocular pressure (IOP). Visual acuity and functional integration were assessed using behavioral tests and immunohistochemistry, respectively. Studies have shown that ES-NP, GP-expressing, transplanted into mice with depleted RGA, which were injected with NMDA, differentiated into a clone of RGA. Improvement in visual acuity was observed 2 months after transplantation (Divya et al. 2017).

The effects of allogeneic Muller cat glia transplantation (fMGSC) with the ability to differentiate into cells expressing RGC markers after RGC removal using *N*-methyl-d-aspartate (NMDA) have also been investigated. In contrast to previous observations in rats, transplantation of hMGSC-derived RGCs into the vitreous body of cats formed aggregates and caused a severe inflammatory response without improving visual function. In contrast, allogeneic transplantation of feline MGSC (fMGSC)-derived RGC into the vitrectomized eye improved the threshold scotopic response (STR) of the electroretinogram (ERG). Despite the functional improvement, the cells did not attach to the retina. Also they did not form aggregates on the peripheral remains of the vitreous, suggesting that the vitreous may represent a barrier to cell attachment to the retina. This has been confirmed by observations that cell scaffolds of compressed collagen and enriched RGC preparations derived from fMGSC facilitate cell attachment. Although the cells did not migrate to the RGC layer of the optic nerve, they significantly improved the STR and photopic negative ERG response, suggesting enhanced RGC function. These results suggest that fMGSCS have a neuroprotective ability that promotes partial restoration of impaired RGC function and indicate that cell attachment to the retina may be necessary for transplanted cells to provide retinal neuroprotection (Becker et al. 2016).

Models of Transient Ischemia

Transient ischemia models cause loss of RGC by occlusion or functional inhibition of the blood vessels supporting the optic nerve and retina, often followed by reperfusion of these vessels – hence named "transient" ischemia. One such model induced retinal ischemia in adult Wistar rats by increasing IOP to 130–135 mmHg for 55 min. Twenty-four hours after the induction of ischemia, BMSCs were injected into the vitreous body. Functional recovery was assessed after 7 days using electroretinography (ERG) measurements of a-wave, b-wave, P2, scotopic threshold response (STR), and oscillatory potentials (OP). Retinal damage and anti-ischemic effects were quantified by measuring apoptosis, autophagy, inflammatory markers, and the permeability of the blood-brain barrier of the retinal. BMSC distribution was qualitatively investigated using real-time fundus images. The introduction of the guiding compound into the vitreous body significantly improved the recovery of ERG a-and b-waves, OP of negative STR and P2, and also reduced apoptosis, as evidenced by a decrease in the levels of TUNEL protein and caspase-3. BMSCs significantly increased autophagy, reduced inflammatory mediators (TNF-α, IL-1β, IL-6), and retinal vascular permeability. BMSCs were preserved in the vitreous and were also observed in the ischemic retina. The results showed that intravitreal injection of PCRS protected the retina from ischemic damage in a rat model. (Mathew et al. 2017).

Li and Zhao (2014) used this model to study the effect of retinal progenitor cell (RPC) transplantation into the subretinal space (SRS) and the superior colliculus (SC) in rats (Li et al. 2014). For transplantation, cultured postnatal rat NPCs were used for 1 day, transfected with an adeno-associated virus-containing cDNA encoding enhanced green fluorescence protein (EGFP). The damage was caused by an increase in intraocular pressure to 110 mmHg for 60 min. The effectiveness of transplantation was evaluated using ERG immunohistochemistry. The transplanted cells survived for at least 8 weeks, migrated to surrounding tissues, and improved ERG responses in the rats with ECP damage. The data obtained showed that RPC transplantation in SRS and SC may be a possible method of cell replacement therapy for retinal diseases (Li et al. 2014).

Transgenic RGC Loss Models

Although they are more challenging to maintain and breed than traditional models, transgenic mouse models using the Cre-Lox system can provide an animal with pure RGC depletion for stem cell research. One example is the Pou4f2 knockout mouse (Brn3b). Pou4f2 is a critical gene for RGC differentiation and is expressed throughout life (Cho et al. 2012). This research group developed a mouse model in which RGC is genetically removed in adult mice. These mice were transplanted with a GFP-labeled progenitors of the embryo's retina. These retinal cells were injected into

the vitreous body of one eye of mice between the ages of 2 and 6 months. Gene expression analysis using an immune tag and the morphology of the optic nerves both visually and using histology were evaluated. The embryonic GFP-labeled RPCs were successfully introduced into the eyes of transgenic mice. Many transplanted RPCs penetrate the ganglion cell layer, and several GFP-labeled cells were found within the optic nerves. At the same time, a significant increase in optic nerve thickness was noted and more connected axons were observed in the retina following RPC injection. The results suggest a new approach to the regeneration of damaged optic nerves. They indicate that a significant number of RPCs toxin differentiate into RGC in the alien environment of the adult retina (Cho et al. 2012).

Model of Experimental Autoimmune Encephalopathy (EAE)

The EAE model primarily models the diseases that cause autoimmune demyelination in the central nervous system, namely multiple sclerosis (MS). Loss of RGC precedes inflammation of the optic nerve in rat models of chronic EAE, whereas, in mice, optic neuritis comes first, and loss of RGC follows. The typical induction time is 1–2 weeks. Current stem cell research on EAE models has focused on neuroprotection via neural or mesenchymal stem cells rather than directly replacing RGCs or their precursors (Pluchino et al. 2009; Lanza et al. 2009).

Models of Hereditary Diseases Associated with Optic Nerve Damage

Retinal degeneration resulting from hereditary diseases such as Leber's hereditary optic neuropathy (LHON) and type I neurofibromatosis (NF1) has no definitive treatment options to date. There are animal models of LHON (Yu et al. 2015). One rotenone-induced LHON model was applied to investigate the protection of visual function in stem cells. Photoreceptor progenitor cells were used, which, according to research, integrate into the ganglion cell layer (GCL). The results of the work were evaluated using magnetic resonance imaging with manganese enhancement, optokinetic responses, and ganglion cell counting. The cultured progenitor cells integrated into the GCL and positive affected maintaining retinal function (Mansergh et al. 2014).

Glaucoma Models

Open-angle glaucoma is the most common glaucoma subtype (Weinreb et al. 2016). It is characterized by an imbalance between the production of water from the ciliary body and its outflow through the trabecular network and uveoscleral pathways. This discrepancy can lead to increased intraocular pressure, resulting in damage to the RGC, as evidenced by pathological changes. Hao and co-authors (2013) studied the

therapeutic effects of executive staff transplantation on vision loss in older rats with glaucoma caused by laser ocular hypertension. The BMSCs contributed to the survival of retinal ganglion cells in the transplanted eyes compared to control eyes. In addition, in swimming tests based on visual cues, rats with a leadership graft performed significantly better. The overall results of the study showed that BMSC transplantation therapy is effective in the treatment of older rats with glaucoma (Hao et al. 2013). An experimental model of photocoagulation of episcleral veins and limbal plexus with an argon laser has been used to study the possibility of retinal stem cell (RSC) transplantation and immunization with glatiramer acetate copolymer-1 (COP-1) copolymer. Rats were immunized with (COP-1) on the same day as glaucoma induction by photocoagulation. RSCs were cultured and transplanted intravitreally a week after laser treatment. The effectiveness of treatment was evaluated based on the expression of brain neurotrophic factor (BDNF), insulin-like growth factor I (IGF-I), and immunohistochemical studies, RT-PCR, and Western blotting were also performed. RGC survival was assessed by TUNEL staining and RGC counts. Their studies showed that the expression of BDNF neurotrophic factors in the IRF-I and RSC/COP-1 group was significantly higher than in the other groups ($P < 0.05$). In addition, the number of apoptotic RGS in the RSC/COP-1 group was markedly lower and the number of RGS in the RSC/COP-1 group was higher. Thus, the researchers concluded that the combined action of RSC and immunization with COP-1 protect RGC against apoptosis in a rat glaucoma model (Zhou et al. 2013).

Harper et al. (2011) used an experimental rat model of chronic ocular hypertension induced by laser cauterization of the trabecular network and episcleral veins in the eyes to study the ability of engineered MSCs to produce and secrete brain neurotrophic factor (BDNF) to protect retinal function and structure after intravitreal transplantation. MSCs expressing BDNF (BDNF-MSC) were transplanted intravitreally. The function of the optic nerve and retina was evaluated using computer pupillometry and ERG; quantitative assessment of optic nerve damage was performed by RGCs counts and by evaluating cross-sections of the optic nerve. After BDNF-MSC transplantation, the eyes retained significantly more retinal and optic nerve functions and showed higher RGC preservation. Interestingly the neurotrophic factors BDNF b-MSCs, transduced by lentiviruses, could persist in the eyes with chronic hypertension and provide functional and structural protection of the retina and optic nerve (Harper et al. 2011).

An ocular hypertension model for the induction of glaucoma, which was performed by cauterizing three episcleral veins (EVC) of the eyes of male Long-Evans rats, has also been utilized. One study investigated the potential of MSCs therapy in this model and deciphered the in vitro effects of MSCs on primary cells within the human trabecular network. BMSCs were labeled with nanocrystals of quantum dots for post engraftment tracing of the cells' fate. The cells were injected in the anterior chamber of the eye for 20 days, with eye pressure monitored twice a week for 4 weeks. At the end of the experiment, the cell distribution in the anterior segment was examined by confocal microscopy on flat corneas. In addition, the effects of the BMSCs-conditioned medium on the primary cells of the trabecular

network were tested in vitro. The work showed a long-lasting and rapid effect obtained following BMSCs transplantation in vivo. Injection of BMSCs into the anterior chamber of the eye in a rat model provides a neuroprotective effect in the pathophysiology of glaucoma. These results demonstrate that BMSCs represent a promising tool to treat ocular hypertension and retinal cell degeneration (Roubeix et al. 2015).

In ocular hypertension induced by an intra-chamber injection of hyaluronic acid into the anterior chamber of rats, the neuroprotective effects of BMSCs and ADMSCs, which were transplanted intravitreally, were investigated. MSCs labeled with green fluorescent protein were transplanted intravitreally 1 week after induction. At the end of the second and fourth weeks, retinal ganglion cells were visualized using the flat retinal attachment method and evaluated using immunofluorescence staining. The results of these studies showed that the number of retinal ganglion cells present was significantly higher when compared to untreated animals. The results of the immunohistochemical analysis showed that a limited number of SCS were integrated into the ganglion cell layer and the inner nuclear layer. The number of cells expressing pro-inflammatory cytokines (interferon-γ and tumor necrosis factor-α) in the group receiving MSCs decreased. On the other hand, the expression of Il-1Ra and prostaglandin E2 receptors had increased. It was concluded that intravitreal MSC transplantation had a neuroprotective effect when the experimental model was reproduced in rats (Emre et al. 2015).

Animal Models of Primary Angle-Closure Glaucoma (PACG)

Primary angle-closure glaucoma, in most cases, is a chronic disease with the gradual appearance of visual symptoms, which differs from open-angle glaucoma only in the degree of angle closure. Possible improvements in visual function by transplanting neural progenitors derived from ESCs were evaluated in a mouse model with glaucoma. Neural progenitors originating from ESCs (ES-NP) were transplanted in a model of preclinical glaucoma (DBA/2 J) with a persistently higher intraocular pressure (IOP). Transplantation experiments in dBA/2D mice showed no significant improvements in visual function, possibly due to the death of both host and transplanted retinal cells, which could be associated with high intraocular pressure. The results showed that strategies for controlling intraocular pressure are necessary for the enhanced survival of transplanted cells in glaucoma (Divya et al. 2017).

This chapter has given an overview of the past and present preclinical research undertaken into MSC-based therapies for ocular diseases. The potential future research directions, complications observed to date, and exciting lines of research have been explored. Several studies have shown the efficacy and safety of MSC therapies in a number of ocular disorders and highlighted the mechanisms of action present within much of the in vitro and in vivo work conducted to date. The eye is a complex organ; replicating human ocular disorders remains a challenge and developing potential therapies is a complex and one of the most intricate and multifaceted challenges, but the one wherein progress is being made.

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Stem Cell for Cartilage Repair

12

Anneh Mohammad Gharravi, Mohammad Reza Gholami, Saeed Azandeh, and Khawaja Husnain Haider

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A. M. Gharravi (✉)
Shahroud University of Medical Sciences, Shahroud, Iran

M. R. Gholami
Kermanshah University of Medical Sciences, Kermanshah, Iran
e-mail: mohammadreza.gholami@kums.ac.ir

S. Azandeh
Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia

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Abstract

Several of the age-related joint diseases such as osteoarthritis lead to chondrocyte degeneration and loss of articular cartilage. Chondrocyte after an injury has restricted capacity to repair. The contemporary clinical treatments such as osteochondral transplantation and arthroplasty procedures have shown serious limitations especially for the treatment of extensive full-thickness cartilage defects. Therefore, alternative therapeutic approaches are warranted. Thus, several cell-based therapies using different stem cells have been proposed as a novel treatment approach for cartilage regeneration and repair. During organogenesis in the embryonic period, chondrocytes originate from mesenchymal progenitor cells. Several studies have reported that treatment using stem cells, especially mesenchymal stem cells, is an optimal intervention for cartilage repair rather than implantation of terminally differentiated cells such as chondrocytes. In this chapter, we have highlighted the use of cell-based therapies for the treatment of injured cartilage and discuss advantages and limitations of different stem cells. We also discuss stem cell-based cartilage tissue engineering as a novel therapeutic strategy for the damaged articular cartilage and discussed in-depth the main components of cartilage tissue engineering such as stem cell source, scaffolds used for their seeding and culture, and types of the bioreactors.

Keywords

Bioreactor · Cartilage · Chondrocytes · Mesenchymal stem cells · Stem cell · Tissue engineering · Transplantation

Abbreviations

AC	Acryloyl chloride
ACI	Autologous chondrocyte implantation
ADSCs	Adipose tissue-derived stem cells
BMDCs	Bone marrow-derived cells
CH	Chitosan
COL	Collagen
CS	Chondroitin sulfate
CSMA	Methacrylated chondroitin sulfate
DJDs	Degenerative-related joint diseases
ECM	Extracellular matrix
GO	Graphene oxide
HA	Hyaluronic acid
OA	Osteoarthritis
OAT	Osteochondral autologous transplantation
OCA	Osteochondral allograft transplantation
OCT	Osteochondral transplantation
PACI	Particulated articular cartilage implantation
PBPCs	Peripheral blood progenitor cells

PC	Pectin-based
PCEC	Polycaprolactone-polyethylene glycol
PCL	Polycaprolactone
PDS	Poly-p-dioxanone
PECA	Poly(ethylene glycol) methyl ether-”-caprolactone-acryloyl chloride
PEG	Poly(ethylene glycol)
PES	Polyethersulfone
PGA	Poly(glycolic acid)
PLA	Polylactic acid
PLCL	Poly(L-lactide-co-”-caprolactone)
PLGA	Polylactic-co-glycolic acid
PLLA	Poly(L-lactide)
PU	Polyurethane
RA	Rheumatoid arthritis
RWVs	Rotating wall vessels
SCPL	Solvent casting and particulate leaching method
SFs	Spinner flasks
SMSCs/SF-MSCs	Synovium fluid-derived mesenchymal stem cells
UCB-MSCs	Umbilical cord blood-derived mesenchymal stem cells
WJ-MSCs	Umbilical cord Wharton’s jelly-derived mesenchymal stem cells

Introduction

Cartilage

Articular cartilage is found at the end of a large area of lining bones in the joint bones. This tissue has important properties, including load-bearing, low-friction, and wear-resistant surface to facilitate joint movement. In terms of structure and composition, the joint cartilage can be divided into three surfaces: The thin superficial (tangential) zone represents approximately 10% to 20% of articular cartilage thickness. The middle with about 40% to 60% of the total cartilage volume contains proteoglycans and thicker collagen fibrils. The deep zone represents approximately 30% of the articular cartilage volume. When articular cartilage is damaged, patients will experience severe pain, inflammation, and some degree of disability (Athanasiou et al. 2010).

Chondrocyte

Articular chondrocytes constitute about 1–10% of the total cartilage volume. They are contained in cavities in the matrix, called cartilage lacunae. Chondrocytes secrete extracellular matrix containing collagen fibers to maintain and sustain the cartilage.

Chondrocytes are separated by a cartilage extracellular matrix consisting of a dense network of collagen and proteoglycan.

Collagen accounts for about 10 to 30% of the dry weight of adult articular cartilage. Proteoglycans, as one of the main components of the cartilage extracellular matrix, bind water and provide the basis for absorbing high compressive loads. Collagen and proteoglycan provide skeletal structure for the articular cartilage and determine cartilage's biochemical and functional properties. Other compounds present in cartilage include salts and little lipid, glycoprotein, and proteins (Darling and Athanasiou 2003; Guilak et al. 2000; Wu et al. 2002).

Collagen

Collagen is the main structural protein in the extracellular matrix found in the articular cartilage. Over 90% of the collagen in the articular cartilage is type I collagen. The arrangement of collagen fibers differs in the layers of articular cartilage; the surface layer is arranged parallel to the cartilage surface. They are oriented almost perpendicular to the bone and cartilage surfaces with a radial arrangement in the inner layers. The articular cartilage also contains other types of collagen fibrillar and globular, such as collagen VI, IX, and V. However, their role does not appear significant in molecular interaction and articular structure (Clark 1990; Deshmukh and Nimni 1973; Eyre and Wu 1995).

Fluid

Water is the most abundant component of articular cartilage; approximately 80% wet weight of cartilage is fluidly associated with the intrafibrillar space within the collagen, in the intracellular space, and pore space of the matrix. Inorganic ions such as sodium, calcium, chloride, and potassium are dissolved in the tissue water. Cartilage is avascular and aneural and receives nutrition by diffusion through the matrix gel. Therefore, fluid plays an essential role in the transport of nutrients and wastes through cartilaginous tissues. It also plays a vital mechanical role (Hardingham et al. 1994).

Chondrocyte Degeneration and Loss of Articular Cartilage

Because articular cartilage, once synthesized, lacks lymphatic or blood supply, several degenerative and age-related joint diseases (DJDs), such as osteoarthritis (OA) and rheumatoid arthritis (RA), lead to chondrocyte degeneration and loss of articular cartilage. Chondrocyte after an injury has restricted capacity to repair. Treatment strategies for DJDs are major challenges of the twenty-first century and vary depending on both cartilage defect and patient factors. At present, both reparative and restorative procedures have been developed for the patients who suffered

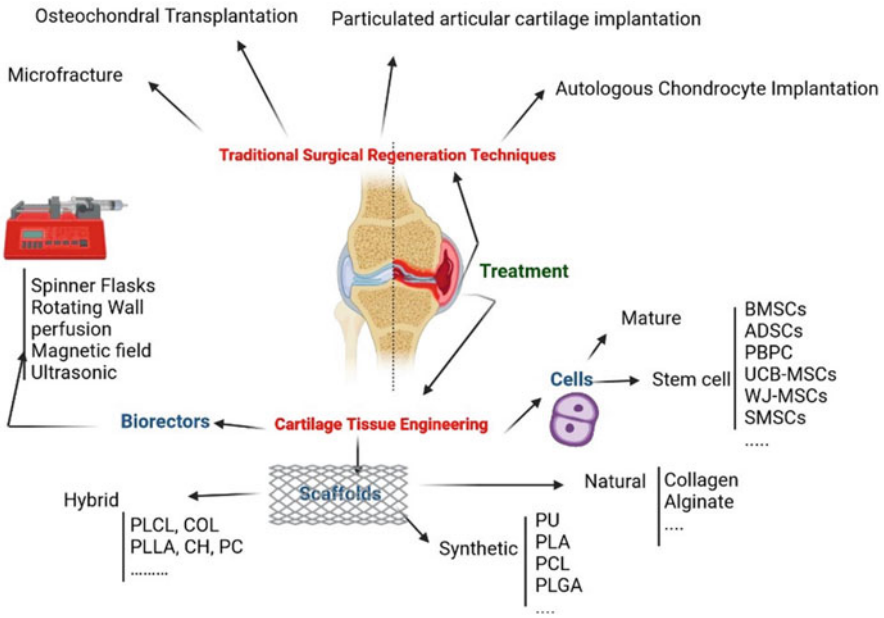

Fig. 1 The articular cartilage defects treatments. Bone marrow-derived cells (BMDCs), adipose tissue-derived stem cells (ADSCs), peripheral blood progenitor cells (PBPCs), umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs), umbilical cord Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs), synovium fluid-derived mesenchymal stem cells (SMSCs/SF-MSCs), CH (chitosan), COL (collagen), PLA (polylactic acid; PU, polyurethane), PLLA (poly(L-lactide)), PLGA (polylactic-co-glycolic acid), PC (pectin-based), PLCL (poly(L-lactide-co-"-caprolactone))

from either partial-thickness or full-thickness cartilage lesions, including microfracture as a reparative technique and osteochondral autografts and allografts in addition to autologous chondrocyte implantation as restorative techniques. In this chapter, we have also highlighted the use of cell-based therapies to treat injured cartilage and discuss the advantages and limitations of different stem cells (Fig. 1). We have also discussed stem cell-based cartilage tissue engineering as a novel therapeutic strategy for damaged articular cartilage and the main components of cartilage tissue engineering, including stem cell source, scaffolds used for cell seeding and culture, and types of the bioreactors.

The Articular Cartilage Defects Treatments

There are several reparative and restorative procedures for patients suffering from partial-thickness or full-thickness cartilage lesions, including microfracture, osteochondral autografts, and allografts, in addition to autologous chondrocyte implantation. Table 1 summarizes the different cartilage defects and the available treatments with their respective advantages and limitations.

Table 1 Advantages and limitations of the articular cartilage defects treatments

Traditional surgical regeneration techniques		Advantages	Limitations
Microfracture		Cost-effective for small ($\leq$6 cm^2) focal defects Reproducible techniques	Limited hyaline repair tissue Fibrocartilage formation Variable repair cartilage volume Partial defect filling Functional deterioration
Osteochondral transplantation (OCT)	Osteochondral autologous transplantation (OAT)	Reproducible and primary treatment strategy Good clinical results at medium long-term follow-up Cost-effective for small focal defects Restoration of hyaline cartilage articulating surface Good chondrocyte survival rate	For younger patients Morbidity in donor site Limited number and graft size and tissue availability Potential chondrocyte apoptosis during graft Lack of cartilage integration Poor matching of graft and host cartilage Congruency
	Osteochondral allograft transplantation (OCA)	Cost-effective Good chondrocyte survival rate Treat large defect Using fully mature articular cartilage Reproducible and primary treatment strategy Good clinical results at medium long-term follow-up Restoration of hyaline cartilage articulating surface	Limited tissue availability Potential chondrocyte apoptosis during graft Risk of disease transmission Potential immunological response Potential chondrocyte apoptosis during graft impaction
Particulated articular cartilage implantation (PACI)	Autologous PACI	Single-stage procedure Treatment of full-thickness cartilage and osteochondral defects medium (10–15 mm) to large (>15 mm) chondral defect Chondrocyte migration into a biomaterial and synthesize new ECM	Active infection at surgical site Diffuse osteoarthritis Malalignment Morbid obesity Patient noncompliance Patients (up to 40 years of age)
	Allogeneic PACI	Overcome the limitations of ACI Immature chondrocytes can better regenerate hyaline-like tissue	Lack long-term follow-up data Risk of disease transmission Limited hyaline repair

(continued)

Table 1 (continued)

Traditional surgical regeneration techniques		Advantages	Limitations
			tissue Potential immunological response
Autologous chondrocyte implantation (ACI)		Arthroscopic or minimally invasive Potential for hyaline cartilage repair tissue Use of autologous cells	Expensive and two-stage procedure Variable repair tissue type: hyaline-like, fibrocartilage, and mixed Limited defect filling and integration Donor-site complications
Tissue-engineered articular cartilage	"Cell-scaffold construct" strategies	High biocompatibility and nontoxicity Provides surface, 3D space, and seed cell support Gradual degradation	Require in vitro expansion of autologous chondrocytes Fibrocartilage formation
	Cell-free strategies	Avoids problems of iatrogenic trauma due to autologous chondrocytes or MSCs harvest Time and expense of cell culture and expansion in vitro	Still in the animal test stage Not in large-scale clinical trials No long-term follow-up data Difficulty in removal of the subchondral bone before implantation
	Scaffold-free strategies	Overcome scaffold-related problems Preserves the chondrocyte phenotypes Provides a natural matrix component Reduce loss of chondrocytes and maintain the defect for a long time	Preclinical, experimental stage A few scaffold-free products available Expensive and no ideal scaffold Requiring a long culture time Complex culturing procedure Expensive

Traditional Surgical Regeneration Techniques

Microfracture

Microfracture is a bone marrow stimulation surgery by making multiple small holes (spaced at 3–4 mm) in the surface of the joint. This is commonly performed for cartilage repair and induces fibrocartilage growth. The procedure basically involves

creating multiple holes in the subchondral region to generate a blood clot containing precursor cell populations derived from the subchondral bone marrow. An important element of the microfracture technique, which is single-stage and technically simple, is the proper preparation of the damaged area. Firstly, a layer of calcified cartilage is removed from the bottom of the lesion and then its edges are evened out. Previous investigations revealed that microfracture has a definite short-term efficacy although it is cost-effective and easy to perform than other methods. However, its sustained treatment benefits are uncertain. The published data show several retrograde changes may occur after microfracture in the subchondral region, including osteophyte formation, cysts, and excessive bone growth which will limit microfracture applications (Galperin et al. 2013; Gudas et al. 2005; Knutsen et al. 2007; Kraeutler et al. 2020; Mithoefer et al. 2006; Polat et al. 2016).

Osteochondral Transplantation (OCT)

Osteochondral transplantation, which is especially useful in active young patients, involves the removal of osteochondral columns (both bone and cartilage obtained from the healthy articular surface) from the non-weight-bearing articular surfaces, such as the femoral trochlear, and their transplantation into repaired articular cartilage defects (Ansah et al. 2007). The goal of OCT is to improve articular function, relieve symptoms, and delay arthroplasty by restoring the articular surface through a biologically active implant. OCT has been shown to be effective in the treatment of medium-sized defects (up to approximately 3–4 cm) (Taşkiran and Ozçelik 2007; Yamashita et al. 1985). There are two types of OCT procedures: osteochondral autologous transplantation and osteochondral allograft transplantation.

Osteochondral Autologous Transplantation (OAT)

In osteochondral autologous transplantation, one or more osteochondral plugs from less weight-bearing joint areas are transplanted as a treatment strategy for small- and medium-sized focal articular cartilage defects (Vogt et al. 2011; Weigelt et al. 2015). This procedure is usually performed in young patients. The scheme of this procedure is to harvest normal cartilage and bone from a nonarticular part of the joint and transplant it into the affected area. The collected cylinders in OAT procedures are larger in size. Therefore, for a typical defect with a diameter up to 9 mm, only one or two of the collected cylinders are sufficient. Consequently, filling the defect with actual articular cartilage and recreating the natural barrier between synovia and subchondral bone may be achieved (Andrade et al. 2016; Hangody and Füles 2003; Sherman et al. 2017; Shimozono et al. 2018).

Osteochondral Allograft Transplantation (OCA)

Osteochondral allograft transplantation (OCA) is a tissue-based articular cartilage and bone reconstruction or replacement method, which comprises the preparation of the cartilage defect, including the debridement of the damaged cartilage for stable healthy borders and fitting and implantation of the osteochondral allograft. An osteochondral allograft is a piece of tissue containing bone and cartilage that is taken from a deceased donor to replace damaged cartilage. This method has emerged

as an important and versatile option in the restoration of articular cartilage, particularly for lesions with both an osseous and a chondral component. The procedure is highly cost-effective (Mistry et al. 2019). Because of the low immunogenicity of articular cartilage, this method of treating cartilage is clinically applicable and does not require immunosuppression. This technique is used primarily in the treatment of large chondral defects (Cook et al. 2014; de Sousa et al. 2015; Langer and Gross 1974; Mankin et al. 1987; McCulloch et al. 2007; Nuelle et al. 2017).

Particulated Articular Cartilage Implantation (PACI)

PACI is a novel treatment modality, which involves autograft and off-the-shelf allogenic juvenile grafts (Riboh et al. 2015; Wixted et al. 2020). By this method, autologous or allogeneic cartilage fragments crushed into small-sized particles are implanted into articular cartilage defects to stimulate chondrocytes' migration and home into the cartilage chips from the extracellular matrix and promote cartilage regeneration (Christensen et al. 2020). Advancement in this regard is the use of PACI to treat chondral and osteochondral defects using platelet-rich plasma scaffolds (Cugat et al. 2021). In this novel approach, platelet-rich plasma scaffold serves as a bioactive component, which induces migration, proliferation, and differentiation of chondrocytes as part of the repair and regeneration process. It is pertinent to mention that given the bioactive molecule-rich composition of platelet-rich plasma, its use may significantly contribute to the ongoing intrinsic repair process (Haider 2017).

Autologous PACI: This procedure is performed by simply cutting autologous cartilage fragments into small size (0.25 to 0.5 mm^3) chips using a scalpel. These pieces are later embedded in scaffold glue before placing them in the debrided defect. Autologous PACI stimulates chondrocyte migration and promotes cartilage regeneration. A commercial product of DePuy company is the cartilage autologous implantation system (CAIS) that results in significantly better international knee documentation committee (IKDC) scores and knee injury and OA outcome scores (KOOS) (Cole et al. 2011; Lu et al. 2006).

Allogeneic PACI: Allogenic PACI involves implanting particulated articular cartilage from a donor as a treatment option for focal articular cartilage defects. Currently, Zimmer's DeNovo NT system as a commercial product derived from allogeneic juvenile cartilage particles results in an extended retention period of up to 45 days in vitro. The repair effect (mixture of hyaline cartilage and fibrocartilage) is reported for 2 years after implantation (Farr et al. 2014; Tompkins et al. 2013).

Autologous Chondrocyte Implantation (ACI): This surgical treatment method involves harvesting chondrocytes from the non-weight-bearing autologous articular surface to treat isolated full-thickness (down to the bone) articular cartilage defects. Autologous chondrocyte implantation (ACI) or mosaicplasty is a two-stage treatment procedure in which chondrocytes are harvested from a patient's joint, expanded in culture, amplified in vitro, and reimplanted over an articular cartilage defect. The American Food and Drug Administration (FDA) has officially approved ACI only as a reparative articular cartilage procedure to treatments of large, grade 3 and 4 chondral defects, up to 10 cm^2. There are three generations of ACI. The first-generation ACI (PACI) involves suturing the autologous periosteum onto the edge of

the cartilage defect and injecting chondrocytes into the defect lacunas. On the other hand, the second generation of ACI (C-ACI) involves the replacement of periosteal tissue with a type I/III collagen membrane, while the third generation of ACI is matrix-induced autologous chondrocyte implantation (MACI) (Brittberg et al. 1994; Giannoni et al. 2005; Kino-oka et al. 2009; McNickle et al. 2009; Peterson et al. 2000, 2002; Rogers et al. 2010; Steinwachs and Kreuz 2007).

Tissue-Engineered Articular Cartilage

The contemporary clinical treatments, such as osteochondral transplantation procedures, have shown severe limitations, especially for extensive full-thickness cartilage defects. Therefore, alternative therapeutic approaches are warranted. Thus, several cell-based therapies using different stem cells have been proposed as a novel treatment approach for cartilage regeneration and repair. There are three categories of cartilage tissue engineering: cell-scaffold constructs, cell-free procedures, and scaffold-free strategies (Caplan and Correa 2011; Harrell et al. 2019; Huang et al. 2016).

"Cell-Scaffold Construct" Strategies

The "cell-scaffold construct" strategy is one of the most commonly used methods in cartilage tissue engineering. Several tissue-engineered articular cartilage technologies based on the "cell-scaffold construct" strategy have been introduced, such as matrix-induced autologous chondrocyte implantation (MACI), BioSeed, Hyalograft, CaReS, NeoCart.

MACI: MACI is a typical tissue engineering procedure, which is a two-part procedure of articular cartilage technology. During MACI, the patient's cells are used to regrow new cartilage. At first, a cartilage biopsy is performed arthroscopically and then planted into a scaffold. Then the construct is implanted into the cartilage defect (Bartlett et al. 2005; Basad et al. 2010; Benthien et al. 2011).

BioSeed®-C: A unit of **BioSeed®-C** is a second-generation implant consisting of approximately 28.8 million patient's autologous chondrocytes embedded in a 3D bioabsorbable gel polymer scaffold. Given that BioSeed®-C employs fibrin glue as the cell carrier to grow the cells on the polyglactin 910/poly-p-dioxanone fleece scaffold. This graft is ideal for 3D homogenous chondrocyte distribution since the chondrocyte phenotype remains differentiated (Huang et al. 2016; Kreuz et al. 2011; Ossendorf et al. 2007).

Hyalograft: Hyalograft is a hyaluronan-based material based on a "cell-scaffold construct" strategy in cartilage tissue engineering. Hyalograft C autograft is an implant consisting of autologous chondrocytes derived from the patient and planted on 2-cm x 2-cm square inserts. The positive clinical results of Hyalograft have shown that it may be a viable therapeutic option for the treatment of acute cartilage lesions (Huang et al. 2016; Nehrer et al. 2009; Schneider et al. 2011).

The Cartilage Regeneration System (CaReS): CaReS uses collagen type I hydrogels as scaffolds for seeding primary autologous chondrocytes. This product has resulted in significantly lower adverse events when compared with ACI in patients during 30-month follow-up after CaReS surgery (Schneider et al. 2011).

NeoCart. NeoCart is an autologous cartilage tissue implant containing autologous chondrocytes embedded in a three-dimensional type I collagen scaffold, resulted similar to that of microfracture surgery, and is associated with greater clinical efficacy (Anderson et al. 2017; Crawford et al. 2012).

NOVOCART® 3D. It is a type I/III collagen biphasic scaffold. The previous investigation revealed that NOVOCART ®3D is an effective method for repairing articular cartilage defects, for treating children and adolescents (Niethammer et al. 2017).

The "cell-scaffold construct" strategy was performed using many types of MSCs with clinical applications, such as bone marrow-derived mesenchymal stem cells (BM-MSCs), adipose tissue-derived stem cells (ADSCs), peripheral blood progenitor cells (PBPCs) and peripheral blood-derived mesenchymal stem cells (PB-MSCs), umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) and umbilical cord Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs), and synovium/synovial fluid-derived mesenchymal stem cells (SMSCs/SF-MSCs).

Cell-Free Strategies

Paracrine release of bioactive molecules and exosomes rich in bioactive molecules constitutes an integral part of the mechanism of MSCs' reparability post-transplantation (Haider and Aziz 2017; Haider and Aramini 2020). Hence, in the recent years, cell-free therapy based on MSCs-derived paracrine factors is now a fast emerging strategy due to its advantages, i.e., avoidance of tumorigenicity, rejection of cell graft, undesired differentiation, etc. (Haider and Aslam 2018). Cell-free procedures for cartilage tissue attempt to repair and regenerate the injured cartilage tissues by recruiting endogenous stem/progenitor cells to participate in the repair (Schüttler et al. 2019). Cell-free strategy can be performed with two methods.

In the first procedure, induction of cartilage regeneration is carried out by in situ endogenous BMSCs using synthetic degradable scaffolds combined with bone marrow stimulation. In the second procedure, to build composite scaffolds of the derivatives secreted by MSCs (i.e., extracellular vesicles, cytokines, and various RNAs) implanted in the cartilage defect areas. Currently, the representative products of cell-free strategies are TruFit scaffolds and MaioRegen scaffolds.

TruFit Scaffolds: The TruFit osteochondral scaffold plug implant is commercially available composed of a poly(lactide-co-glycolide) copolymer, 10% calcium sulfate, polyglycolide fibers, and surfactant for the treatment of chondral and osteochondral defects. Investigations indicated a clear improvement of the clinical symptoms and slowing joint degeneration in the case of focal osteochondral (Azam et al. 2018; Barber and Dockery 2011; Bugelli et al. 2018; Joshi et al. 2012).

MaioRegen Scaffold: The MaioRegen scaffold is a three-layer biomimetic scaffold consisting of collagen I and hydroxyapatite. Studies have shown that it has a reliable medium-term effect on repairing articular cartilage defects. MaioRegen scaffold has been proposed for in situ cartilage regeneration. MaioRegen, with a porous composite structure, mimics the whole osteochondral anatomy. It is a nanostructured biomimetic and bioresorbable implant, which has three different layers (superficial, intermediate, and lower). In the superficial layer with only type I

collagen (100% type I collagen) mimicking smooth surface, it reproduces the articular surface. The intermediate layer has collagen type I and 40% hydroxyapatite composite (60% type I collagen and 40% hydroxyapatite), while the lower layer has 30% collagen type I and 70% hydroxyapatite that reproduces the composition of the subchondral bone (Berruto et al. 2014; D'Ambrosi et al. 2019).

Scaffold-Free Strategies

In recent year, the scaffold-free strategies and products have gained popularity for use in the clinic as the process is similar to the native cartilage development. These novel products can completely regenerate continuous, homogeneous, and healthy cartilage layers, because it mimics the elements of developmental processes underlying these tissues. This method needs important consideration, such as cell sourcing, stimulation of tissue-specific extracellular matrix (ECM) production, and tissue organization (Park et al. 2019; Yamashita et al. 2015). A few scaffold-free products are currently being used in clinical applications.

Chondrosphere (Spherox): Chondrosphere is a 3D autologous chondrocyte transplantation product that comprises spheroids in suspension developed from autologous chondrocytes, which indicated for the repair of symptomatic articular cartilage defects. The spheroids are formed from the patient's undamaged articular condyles. Human chondrocytes undergo three steps for the production of spheroids harvesting from healthy articular cartilage, cultivation for 8–10 weeks in vitro, and condensing into spheroids (chondrospheres). The three-dimensional spheroids are then transplanted into the defect. In literature, the recommended dose of spheroids of human autologous matrix-associated chondrocytes was 10–70 spheroids/cm^2 (Armoiry et al. 2019; Becher et al. 2017).

Common Cell Types for Articular Cartilage Regeneration

During organogenesis in the embryonic period, chondrocytes originate from mesenchymal progenitor cells. Several studies have reported that treatment using stem cells, especially MSCs, is an optimal intervention for cartilage repair rather than implanting terminally differentiated cells such as chondrocytes. Various cell types from diverse tissue sources have been characterized as possible candidates for cartilage repair (Ma et al. 2018). The reparability of these cells has been assessed with or without manipulation using various scaffolds for the delivery, as discussed in the following sections (Huang et al. 2018a). Table 2 summarizes the advantages and limitations of the use of common cell types for articular cartilage regeneration.

Mature Cells

Autologous chondrocytes were one of the first tissue cells for tissue engineering applications to regenerate the articular cartilage surface. Autologous chondrocytes can be used for larger chondral lesions, where the subchondral bone plate remains

Table 2 Advantages and limitations of the use of common cell types for articular cartilage regeneration

Cell types		Advantages and limitations													
		IR	DT	CD	SRA	NC	IP	MDS	EC	RF	DWA	T	LSCT	CE	Other
Autologous chondrocytes		–	–	+	+	L	+	+	–	+	+	–	+	–	Chondrocyte phenotype
	BMSCs/ BMDCs	+	+	+	+	L	+	+	–		+	+	+	–	Variable in different individuals
MSCs	**ADSCs**	+	+	+	+	H	Small		–		+	+	+	–	Variable in different individuals
	PBPCs/PB-MSCs	+	+	+	–	L	–	–	–		+	+	–		Variable in different individuals
	UCB-MSCs/ WJ-MSCs	–	+	+	+	H	–		–		–	+	–	+	Resistant to senescence
	SMSCs/SF-MSCs	–	–	+	+	L	+	–	–		+	+	–	–	
ESCs		–	–	+	+	L	+	+	+		–	+	–	–	Capacity to differentiate into three germ layers
iPSCs		–	–	+	+	Low	+	+	–		–	+	–	–	Capacity to differentiate into three germ layers

CD chondrogenic differentiation, *CE* cost-effectiveness, *DT* disease transmission, *DWA* decreased with age, *EC* ethical controversy, *IP* invasive procedures, *IR* immunorejection, *LSCT* large-scale clinical trials, *MDS* morbidity at the donor site, *NC* number of cells, *RF* regenerate fibrocartilage, *SRA* self-renewal ability, *T* tumorigenicity

intact. The previous investigation revealed indications of autologous chondrocyte implantation for larger and full-thickness defects (e.g., 2–10 cm^2) in younger patients, to treat injuries that have failed debridement or other cartilage repairs after a preceding bone graft for deeper lesions. But in the patient with inflammatory arthritic condition, kissing lesions (in particular, outer bridge grades 3–4 on the opposing surface) and reluctance to cooperate with postoperative rehabilitation should be avoided. Although several investigations showed that autologous chondrocytes have potential in cartilage tissue engineering applications, many restrictions limit the application of autologous chondrocytes, such as limited number of cells (Brittberg et al. 1994; Giannoni et al. 2005; Kino-oka et al. 2009; McNickle et al. 2009; Peterson et al. 2000, 2002; Rogers et al. 2010; Steinwachs and Kreuz 2007).

Stem Cells

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) constitute the heterogeneous stromal multipotent adult stem cells compartment of the bone marrow (BM) with diverse properties, including plastic adherence, clonal expansion by self-renewal, and tri-lineage differentiation potential. MSCs have capacity multipotentiality, and under a conducive set of conditions, MSCs can transdifferentiate into at least three cell lineages, adipocytes, osteoblasts, and chondrocytes, besides others, i.e., myofibroblasts. MSCs can be isolated from several body tissues, such as the placenta, umbilical cord, skeletal muscle, synovium, synovial fluid, and adipose tissue (Bianco and Robey 2015; Frisbie and Stewart 2011; Kim et al. 2014; Lee et al. 2007; Li et al. 2016). There are currently many types of MSCs with underexplored and even unexplored potential of clinical utility. However, some of these cells have already progressed to advanced stages of clinical trials for the treatment of various diseases, i.e., bone marrow-derived MSCs, adipose tissue-derived MSCs (AD-MSCs), peripheral blood-derived stem/progenitor cells, umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs), umbilical cord Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs), and synovium/synovial fluid-derived mesenchymal stem cells (SMSCs/SF-MSCs) (Le et al. 2020).

BM-MSCs

BM-derived MSCs are an integral part of the HSCs' niche in vivo. Based on their surface marker expression, they are positive for CD90, CD44, CD70, etc., but negative for the expression of hematopoietic stem cell-specific markers, i.e., CD34, CD45, etc. (Haider and Ashraf 2005). The International Society for Cell Therapy (ISCT) has suggested standardized criteria to define human MSCs which include (1) adherence to plastic surface under standard culture conditions; (2) expression of CD73, CD90, and CD105 and lack of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 membrane surface molecules; and (3) tri-lineage differentiation potential to adopt osteoblasts, adipocytes, and chondroblasts under a defined

set of culture conditions in vitro (Haider 2018). Although they are available in small numbers in the BM, BM-MSCs are easy to harvest and expand in vitro undifferentiated to achieve a relatively high concentration that is enough for use. Given their robust nature, they can be genetically manipulated or preconditioned to enhance their paracrine activity as well as survival and differentiation potential (Haider et al. 2008, 2010; Kim et al. 2009, 2012). Hence, BM-MSCs are the most well-characterized, better studied, and commonly used in the field of articular cartilage regeneration.

Published data have demonstrated that BM-MSCs possess the capability of differentiating into cartilage and, hence, repair articular cartilage defects. These data have been reported in numerous preclinical as well as clinical studies and showed that BM-MSCs significantly improved patients' quality of life after treatment (Li et al. 2018; Nejadnik et al. 2010; Park et al. 2015; Wakitani et al. 2007).

Various research groups have demonstrated that autologous BM-MSCs seeded on a collagen gel or combined with platelet-rich fibrin glue scaffolds could significantly improve the symptoms of cartilage defects (Jung et al. 2009; Berninger et al. 2013; Araki et al. 2015). No adverse events have been reported, demonstrating the safety of and efficacy of scaffold-based delivery of BM-MSCs. Several investigations have reported the effects of bioactive factors on BM-MSCs' chondrogenic differentiation (Fortier et al. 2011; Patel et al. 2019). For example, TGF-β3 activated the downstream TGF-β3/Smad signaling pathway and promoted MSCs' chondrogenic differentiation (Ng et al. 2008). Bian et al. have designed an implantable construct by co-encapsulating MSCs and TGF-β3 containing alginate microspheres in HA hydrogel for cartilage repair (Bian et al. 2011). Subcutaneous implantation of the construct into nude mice resulted in a superior cartilage matrix formation, attributed to controlled local delivery of TGF-β3. In another study, TGF-β3, through the activation of MAPK and Wnt signaling pathway, induced gene expression of SOX9, COL II, and ACAN and proteoglycan synthesis (Tuli et al. 2003). Although the role of TGF-β3 in chondrogenic differentiation of MSCs is well-established, the approach is not without its drawback that includes inefficiency of the protocol and instability of chondrogenic phenotype.

Besides TGF-β3, bone morphogenetic proteins (BMPs) superfamily, including BMP-2, BMP-4, BMP-6, and BMP-7, have shown to significantly enhance MSCs' chondrogenic differentiation (Taipaleenmäki et al. 2008; Scarfì 2016). BMPs function by the activation of Smad-dependent and Smad-independent signaling pathways that involve activation of mitogen-activated protein kinases (ERK, p38MAPK, JNK) (Carreira et al. 2015). On the same note, another TGF superfamily, the addition of FGF-2 during the expansion of BMSCs, alters MSCs' surface marker distribution and chondrogenic differentiation potential and increased GAG/DNA contents (Hagmann et al. 2013).

Bioactive melatonin attenuates IL-1β-induced activation of NF-kB pathway and rescued interleukin 1b-impaired chondrogenic potential of BM-MSCs. In similar studies, melatonin increased gene expression of SOX9, RUNX2, SERPINB2, and SERPINA9 and enhanced the accumulation of GAG, COL II, and COL X (Gao et al. 2014; Granados-Montiel and Cruz-Lemini 2021; Hu and Li 2019). Treatment with

chondroitin sulfate promoted BM-MSCs' chondrogenic differentiation and also inhibited chondrocyte hypertrophy (Varghese et al. 2008), while ghrelin treatment upregulated COL II, SOX9, and ACAN expression and enhanced the collagen and GAG accumulation in vitro through activation phosphorylation of ERK1/2 and DMNT3A pathway. These molecular changes improved the cartilage repair effect of BMSCs in vivo through SHH pathway activation (Li et al. 2012). Atractylenolides increase SOX9, COL II, and ACAN gene expression (Li et al. 2012).

Several investigations have also reported the effects of physical stimulation of BMSCs in terms of supporting chondrogenic differentiation, such as vibration and compression. These methods of physical stimulation include mechanical methods (i.e., cyclic strain, fluid shear stress), electrical and magnetic field exposure, ultrasound and shock-wave treatment, and substrate stimuli (Huang et al. 2018). Vibration through activation of Wnt/b-catenin pathway promoted BM-MSCs' chondrogenic differentiation and inhibited hypertrophic differentiation. Tensile stimulation improved chondrogenic phenotype of MSCs (Hou et al. 2020; Xie et al. 2019).

Compression stimulation functions by TGF-β/activin/nodal pathway and suppresses BMP/GDP and integrin/FAK/ERK pathways. These molecular changes enhanced BM-MSCs' chondrogenic differentiation and suppressed chondrocyte hypertrophy and fibrocartilage formation (Xie et al. 2019). Microgravity suppressed IHH and SHH pathways and attenuated their chondrogenic differentiation and chondrocyte hypertrophy as well as aging of MSCs by increasing the expression of COL II and SOX9 (Xie et al. 2019; Zhang et al. 2015).

WJ-MSCs

Similar to BM-MSCs, WJ-MSCs have multi-lineage differentiation potential. Investigations have demonstrated that WJ-MSCs can sustain undifferentiated self-renewal in vitro for more than ten generations. WJ-MSCs express surface markers similar to the phenotype of BM-MSCs. Although WJ-MSCs are allogenically sourced, they are weakly immunogenic; they resemble BM-MSCs in terms of paracrine activity (Marino et al. 2019), and hence, they are considered an excellent cell source in tissue engineering, especially when cells have to be allogenically sourced (Voisin et al. 2020). Interestingly, studies have demonstrated that immune-related molecules (i.e., B7-H3/CD276 and HLA-E) characterized in undifferentiated MSCs were also expressed in the differentiated progeny of WJ-MSCs and suggest that WJ-MSCs-derived cells may maintain their immunoprivileged status post differentiation (La Rocca et al. 2013). Similar observations have also been reported recently by Voisin et al., who have demonstrated that hypo-immunogenicity is sustained and remains unchanged during the process of differentiation. A head-to-head comparison of BM-MSCs and WJ-MSCs has shown that the latter's immunoprivileged properties and stemness were much superior as compared to BM-MSCs (Li et al. 2017).

Human WJ-MSCs co-cultured with primary cartilage cells on an acellular cartilage ECM scaffold significantly enhance the biomechanics and composition of the neo-tissue post engraftment into the articular cartilage defect area in a caprine

experimental model (Zhang et al. 2020). The differentiated WJ-MSCs showed a stable chondrogenic phenotype in the biomimetic cartilage microenvironment. More interestingly, the neo-tissue was more similar to native cartilage during 9 months of follow-up.

Various strategies have been adopted to enhance the rate of differentiation. One such strategy combines WJ-MSCs and pulsed electromagnetic field (PEMF), which induce an early and higher rate of WJ-MSCs differentiation toward cartilaginous tissue (Esposito et al. 2013). On the same note, a study aimed to evaluate the usefulness of two commercially available hyaluronic acid (HA)-based hydrogels, HyStem and HyStem-C, for the cultivation and chondrogenic differentiation of WJ-MSCs showed that the cells had some degree of chondrogenic potential in both the hydrogels (Aleksander-Konert et al. 2016). The use of advanced hydrogels with tailored properties has been excellently reviewed by Wei et al. (Wei et al. 2021).

UCB-MSCs

Human UCB-MSCs (hUCB-MSCs) offer a promising cell source for in vivo repair of cartilage defects. Cord blood is collected from the umbilical cord, and placenta UCB-MSCs have several advantages over BM-MSCs and AD-MSCs, including noninvasive availability, high proliferative potential, presence of pluripotent cell population, low immunogenicity, and chondrogenic potential. In recent years, the co-culture of hUCB-MSCs and chondrocytes in vitro has been shown to promote hUCB-MSC chondrogenesis (Zheng et al. 2013). A direct comparison between BM-MSCs, AD-MSCs, and UCB-MSCs revealed that UCB-MSCs had the highest proliferation capacity, clonality paracrine activity, and tolerance to aging (Jin et al. 2013). It was observed that the ratio of co-cultured cells and the presence of IGF-1 in the differentiation culture significantly enhanced their chondrogenic potential. More interestingly, the defined ratio between the two cell types resulted in the expression of the cartilaginous cellular matrix (Zheng et al. 2013).

Besides co-culture, various strategies have been developed to enhance the rate of chondrogenic differentiation. Thrombospondin-2 secreted by UCB-MSCs as part of paracrine activity triggers chondrogenic differentiation of chondroprogenitor cells (Jeong et al. 2013). Molecular studies showed autocrine activation of the Notch signaling pathway during chondrogenic differentiation of UCB-MSCs and attenuated their hypertrophic differentiation (Jeong et al. 2015). In a recent study, Jing et al. have exploited the preconditioning approach during which the authors treated UCB-MSCs with kartogenin before TGF-β3 induction. The authors observed activation of the JNK/RUNX1 signaling pathway with concomitant suppression of the β-catenin/RUNX2 pathway. The preconditioned cells showed accumulation of extracellular matrix and chondrogenic gene expression of SOX9, COL II, and ACAN (Jing et al. 2019). Similar data have also been reported by Zhao et al. that have also shown the superiority of concomitant use of kartogenin preconditioning and TGF-b3 induction for chondrogenesis in UCB-MSCs (Zhao et al. 2020). Some recent advances in the use of kartogenin for chondrogenic differentiation of UCB-MSCs are their use with hydrogels and collagen nanofibers (Yin et al. 2017; Fan et al. 2020). Another study has reported that a combination of allogeneic UCB-MSCs and

HA hydrogel is a safe and effective treatment option for large osteochondral defects (Park et al. 2017).

SMSCs/SF-MSCs

The synovial fluid (SF) is a novel source of MSCs (SF-MSCs) for cartilage repair (Li et al. 2020). SF-MSCs can be harvested during arthrocentesis, arthroscopy, or knee surgery with chondrogenic potential. Like BM-MSCs and UCB-MSCs, SF-MSCs have shown significant chondrogenic differentiation capacity (Neybecker et al. 2020). A recently published systemic review including 20 studies (4 using human- and 16 using animal-sourced SF-MSCs) has concluded that irrespective of the source, harvesting protocol, delivery method, etc., SF-MSCs successfully and effectively repaired focal cartilage damage/defects (Kendrick et al. 2019). There is mounting evidence in the published data that SF-MSCs may have superior chondrogenic potential compared with MSCs from other tissue sources (Bami et al. 2020). Molecular studies have revealed the activation of RhoA/ROCK involved in TGF-β3-induced chondrogenic differentiation of rat SF-MSCs through interaction with the Smad pathway (Xu et al. 2012). A study conducted on bovine synovium-derived progenitor cells cultured in 3D alginate hydrogel demonstrated that BMP-2 could induce these cells to express chondrocyte-specific genes, Sox9, type II collagen, and aggrecan (Park et al. 2005). In vitro cartilage formation of composites of SF-MSCs with collagen gel effectively induced chondroitin sulfate and mRNA expression for cartilage-related genes that demonstrated cartilage maturation (Yokoyama et al. 2005). Also, addition of dexamethasone in the combination of TGF-β and BMP2 significantly enhanced in vitro chondrogenesis of SF-MSCs (Shirasawa et al. 2006).

Adipose Tissue-Derived MSCs

Adipose tissue contains stem cells that have the capacity to differentiate into cartilage. ADSCs' effective reparability and regeneration potential of cartilage have been reported in a variety of animal models. Therefore, they represent a promising, minimally invasive, nonsurgical source of cells for cell-based therapy of cartilage defects. ADSCs obtained from lipoaspirates have immunosuppressive properties and low immunogenicity (Simona et al. 2020; Huaman et al. 2019). Moreover, they actively secrete trophic factors as part of their paracrine activity that actively contributes to the therapeutic and regenerative potential of the cells in a wide range of applications (Linero and Chaparro 2014; Dabrowski et al. 2017).

Treatment with bioactive factors of BMPs family, such as BMP-2, BMP-4, BMP-6, and BMP-7, enhances ADSCs' chondrogenic differentiation (Taipaleenmäki et al. 2008). However, a recent study has reported that BMP-2 outperforms BMP-6 in promoting chondrogenesis using canine adipose tissue-derived MSCs (Teunissen et al. 2021). The authors also reported a direct comparison of canine AD-MSCs with BM-MSCs to demonstrate the former were inferior in chondrogenic differentiation. However, both the cell types expressed common surface markers. An earlier study has reported that this weakness of AD-MSCs may be compensated by higher dose treatment of pro-chondrogenic growth factors to achieve a comparable rate of chondrogenic differentiation rate (Kim and Im, 2009). On the same note, kartogenin

treatment significantly promotes chondrogenic differentiation, suppresses chondrocyte hypertrophy in ADSCs' fibrin scaffold (Valiani et al. 2017), and enhances the expression of chondrocyte redifferentiation markers (Zhang et al. 2021). In another interesting study to enhance their chondrogenic differentiation, ADSCs from adipose were transduced for ectopic expression of Sox-9 with a retroviral vector encoding the Sox-9 gene in vitro. The transduced cells were engineered for the chondrocyte-like cell differentiation by 3D culturing in alginate beads and TGF-β treatment. Ectopic expression significantly increased the chondrogenic potential of the cells (Yang et al. 2011). These studies provide strong evidence that AD-MSCs can be strong candidates for use in the clinical perspective.

PB-MSCs

Peripheral blood (PB) is yet another potential source of chondrostem/progenitor cells that can be used for cartilage repair and regeneration. PB-derived stem/progenitor cells can be obtained by a minimally invasive procedure without general invasiveness and complications associated with MSCs harvesting from many other tissues, such as hemorrhage, chronic pain, neurovascular injury, etc. PB-derived stem/progenitor cells secrete circulating cytokines, which puts them at par with MSCs from other tissue sources for autologous cell-based therapy. It is generally perceived that PB-derived stem/progenitor cells positively influence MSCs' emigration from the BM. A direct comparison of PB-derived MSCs and BM-MSCs has revealed that the two cell types were similar in their biological characteristics, but the chondrogenic potential of BM-MSCs was superior to PB-MSCs (Lotfy et al. 2019).

Several studies conducted with PB-derived stem/progenitor cells for cartilage repair and regeneration have reported that autologous PB-derived stem/progenitor cells are safe for use in vivo. In a recent study, it has been demonstrated that quality of life improvements measured by WOMAC and KOO scores increased after successful regeneration of articular cartilage during early osteoarthritic disease (Turajane et al. 2013). Cells derived from mobilized peripheral blood showed similar biological characteristics in chondrogenesis as MSCs from the NM. In some studies, PB-derived stem/progenitor cells have shown even better chondrogenic differentiation potential than BM-MSCs in vitro (Fu et al. 2014). Hypoxic culture of PB-derived stem/progenitor cells seeded on HA and collagen scaffolds significantly enhanced the expression of pro-chondrogenic cells in the seeded cells (Bornes et al. 2015).

Scaffolds for Cartilage Treatment

A recent systematic review has shown that cellular scaffolds are undoubtedly more effective than the noncellular scaffolds (Pot et al. 2017). However, the nature of the scaffold material remains a significant determinant of the outcome because the scaffold type and biomaterials in cartilage tissue engineering should provide a suitable and conducive microenvironment mimicking the natural microenvironment in the articular cartilage. This is important for the cells to sustain their biological and differentiation characteristics during culture and post engraftment in the tissue

regeneration process. Therefore, ideal scaffolds for cartilage tissue engineering should have the following essential features that include but are not restricted to improved cell attachment behavior, biodegradable, noncytotoxic, biocompatible, highly porous, support proliferation and differentiation, flexible and elastic, and nonantigenic. Currently, matrix materials suitable for chondrocytes can be made using synthetic or natural polymers or both (hybrid scaffolds). Table 3 summarizes natural scaffolds approved for cartilage tissue engineering for medical use, while Table 4 summarizes the synthetic and hybrid scaffolds for cartilage tissue engineering.

Commercially available natural polymers, i.e., chitosan, collagen, alginate, silk fibroin, hyaluronan, and gelatin, exhibit excellent tissue compatibility, little toxicity, and facile biodegradation (Jiang et al. 2020; Li et al. 2020). Despite these advantages, however, some of the common drawbacks of natural materials include their weak mechanical properties and unstable degradation rate, which significantly limit their application in cartilage tissue engineering (Xia et al. 2021). To overcome these limitations of the natural polymers, synthetic materials such as polyurethane (PU), polylactic

Table 3 Natural scaffolds approved for medical use for cartilage tissue engineering

Base	Scaffold	Product (company)	Properties
Collagen	**Bovine type I collagen**	NeoCart® (Histogenics)	Two-step procedure Expanding chondrocytes into scaffolds Incubation in the tissue engineering processor
	Type I/III	Chondro-Gide (Geistlich)	One-step procedure The first described matrix for the ACI method
	Type I/III	ACI-Maix™ (MACI)	Two-step procedure Expanding chondrocytes into scaffolds Implantation into the patient
	Type I collagen, chondroitin sulfate	NOVOCART® 3D—Aesculap Orthopaedics (BBraun)	Two-step procedure A sponge form
	Type I collagen gel	CaReS® (Arthrokinetics)	Two-step clinical procedure Mixing of isolated autologous chondrocytes Fluid matrix A fibrin glue
Agarose and alginate	**Agarose and alginate**	Cartipatch® (Xizia Biotech)	Two-step method The cylindrical scaffold of a single layer of hydrogel with expanded cartilage cells
Hyaluronic acid	**Benzyl ester of hyaluronic acid**	Hyalofast® (Anika)	One-step procedure bioresorbable Nonwoven structure
	Hyaluronic acid	CARTISTEM® (Medipost)	Allogeneic human umbilical cord blood (hUCB)-derived MSCs and HA hydrogel

Table 4 Synthetic hybrid scaffolds for cartilage tissue engineering. (Kreuz et al. 2011; Christensen et al. 2012; Theodoridis et al. 2019a; Tsai et al. 2015)

Type	Base	Scaffold	Source/animal model	Properties
Synthetic	PGA	Chondrotissue® (Bio-Tissue)	Platelet-rich plasma and bone marrow concentrate	Provide one-step cartilage repair method
	PU	Spongy PU scaffold	Chondrocytes, human MSC	Biodegradable
	PCL	NSP-PCL scaffold	Rabbit articular chondrocytes	In vitro and in vivo studies results
	PES	Polysulfonic scaffold	Rabbit model and human articular chondrocytes	Effective in repairing articular cartilage defects
	PLLA	PLLA-100 scaffolds	Human articular chondrocytes	Promote the secretion of chondrogenic genes
	PLCL	PLCL-2 scaffold	Rabbit articular chondrocytes and mice model	Maintain mechanical integrity of chondrocyte
Hybrid	PGA, HA	Chondrotissue® (Bio-Tissue)	Platelet-rich plasma and BM concentrate	For the one-step cartilage repair method
	PLGA, COL	IC scaffold	Bovine articular chondrocytes (BACs) and mice model	Promote gene expression, chondrocyte proliferation, and regeneration of cartilage tissue with high mechanical properties
	Gelatin, PCEC, TGF_1	Gel/PCEC-TGF_1 hydrogel scaffold	Human adipose tissue (AD)-MSCs	The potential for the growth and differentiation of h-AD-MSCs
	PLCL, COL	PLCL-COLI	Rabbit articular chondrocytes	Controlled structure, good biocompatibility, elasticity, and mechanical properties
	C2C1H scaffold	PLA, COL, CH	Bovine articular cartilage chondrocytes	High porosity, good mechanical strength, and interconnected pore network
	PLGA, ECM	ECM-PLGA scaffold	Rat mesenchymal stem cells (MSCs) and rat model	Improve attachment, proliferation, and differentiation of the MSCs
	PCL, COL	PCL/COL1	Pig articular chondrocytes and nude mice model	High porosity and repetitive pore structure
	PLLA, CH, PC	CH/PLLA/PC scaffold	Rabbit articular chondrocytes	Suitable for cartilage tissue regeneration

(continued)

Table 4 (continued)

Type	Base	Scaffold	Source/animal model	Properties
	PLCL, CH	Chitosan-modified PLCL scaffold	Pig articular chondrocytes	Biodegradable, with high porosity, good mechanical strength, and interconnected pore structure
	CSMA, GO MPEG-PCL-AC (PECA)	CSMA/PECA/GO (S2) scaffold	Rabbit articular chondrocytes	Scaffold with an appropriate structure with biological components

List of Abbreviations: AC, acryloyl chloride; CH, chitosan; COL, collagen; CS, chondroitin sulfate; CSMA, methacrylated chondroitin sulfate; ECM, extracellular matrix; GO, graphene oxide; HA, hyaluronic acid; PC, pectin-based; PCEC, polycaprolactone-polyethylene glycol; PCL, polycaprolactone; PDS, poly-p-dioxanone; PECA, poly(ethylene glycol) methyl ether-"-caprolactone-acryloyl chloride PEG—poly(ethylene glycol); PES, polyethersulfone; PGA, poly(glycolic acid); PLA, polylactic acid; PLCL, poly(L-lactide-co-"-caprolactone); PLGA, polylactic-co-glycolic acid; PLLA, poly(L-lactide); PU, polyurethane; SCPL, solvent casting and particulate leaching method

acid (PLA), polycaprolactones (PCL), and poly(lactide-co-glycolide) (PLGA) with unique properties including plasticity, degradation rate, and mechanical characteristics have been introduced. Synthetic materials allow forming into desired shapes and improved control over mechanical and structural features besides having resorbable properties (Joshi et al. 2012). However, synthetic materials are very costly and have weak cell-adhesive ability (Wei and Dai, 2021). Therefore, in-depth investigations are currently underway to develop hybrid materials such as gelatin/polycaprolactone-polyethylene glycol (Gel/PCEC-TGF1) (Huang et al. 2016; Irawan et al. 2018; Wasyłeczko et al. 2020).

Bioreactors

Currently, the most common types of cartilage tissue bioreactors are the spinner flasks, rotating wall vessels, and perfusion systems, which have been developed in an attempt to provide necessary features, such as enhancing the expansion of seeded cells, promoting the exchange of nutrients and oxygen, and providing appropriate physicochemical stimuli (Mabvuure et al. 2012; Silva Couto et al. 2020). Table 5 summarizes common types of bioreactors for cartilage tissue engineering.

Spinner Flasks

Spinner flask is a cylindrical culture system that can create fluid convection and hydrodynamics to enhance the efficiency of nutrient delivery and seeding of cells in the scaffold that result in chondrogenic differentiation in vitro and subsequent

Table 5 Common types of bioreactors for cartilage tissue engineering

Type of bioreactor	Cell source	Study design	Results
Spinner flasks (SF)	Chondrocytes	Alginate gel beads with chondrocytes	Improvement of the GAG quantification and relevant gene expression
	hADSCs	Cellular metabolic response to dynamic loading	Enhancing chondrogenic differentiation of hADSCs
Rotating wall vessels (RWVs)	hADSCs	Chitosan/gelatin hybrid hydrogel and subsequent dynamic loading	Enhancing proliferation and matrix secretion
	RCS	Culture of cell pellets	Influence the expression of LRP4/5/6 in chondrocytes
Perfusion bioreactors (PB)	hADSCs	D-printed PCL scaffold-seeded cells	Uniform distribution of the cells within the scaffold
	hBM-MSCs	hBM-MSCs cultured in the perfusion bioreactor	Generated a homogeneous hypertrophic cartilage
Magnetic field bioreactors (MFB)	hBM-MSCs	Scaffold-free hBM-MSCs sheets in response to variable magnetic fields	Did not affect cartilage formation
Ultrasonic bioreactors (USBs)	Chondrocytes	Chondrocyte-seeded scaffolds	Impacted cell proliferation and depth-independent cell population density
	Chondrocytes	Studying the response of cells to ultrasonic stimulation	Influence cell proliferation, viability, and gene expression

List of Abbreviations: *hAD-MSCs* Human adipose tissue-derived MSCs, *hBM-MSCs* human bone marrow-derived MSCs, *MSCs* mesenchymal stem cells, *PB* perfusion bioreactors, *MFB* magnetic field bioreactors, *RWVs* rotating wall vessels, *SFs* spinner flasks, *USBs* ultrasonic bioreactors

chondrogenic formation in vivo (Mabvuure et al. 2012; Rauh et al. 2011; Yoon et al. 2012).

Rotating Wall Vessels

These vessels consist of a pair of concentric cylinders: rotating outer and inner cylinders that are static and used to exchange gases that create a microgravity environment and provide controlled oxygen transport, as well as low shear forces and turbulence (Nordberg et al. 2019; Zhu et al. 2017).

Perfusion Bioreactors

The perfusion bioreactors consist of a pump, culture media reservoir, tubing circuit, and a perfusion cartridge that holds the scaffolds and effectively infuses the medium into the scaffold. Perfusion bioreactors use a pumping system to feed the medium

directly to the scaffolds (Gharravi 2019; Gharravi et al. 2012, 2014, 2016; Pigeot et al. 2020; Sharifi and Gharravi 2019; Theodoridis et al. 2019b).

Magnetic Field Bioreactors

The magnetic field bioreactors (MFB) can meet the requirement of sterile culture conditions by contactless culture. Most of them consist of one group of permanent magnets that influence the behavior of cells through static or dynamic magnetic field strengths (Brady et al. 2014; De Mattei et al. 2004; Dikina et al. 2017; Dobson et al. 2006; Jaberi et al. 2011).

Ultrasonic Bioreactors

Low-intensity continuous ultrasound (US) has been shown to influence cell growth and modulate the expression of chondrocyte-specific genes. The traditional ultrasonic bioreactor (USB) is realized by adding an ultrasonic processing system (Guha Thakurta et al. 2014; Subramanian et al. 2013; Whitney et al. 2012).

Conclusion

None of the currently available methods, microfracture as reparative technique and osteochondral autografts and allografts, in addition to autologous chondrocyte implantation as restorative techniques, can be said not to have any disadvantages. Stem cell-based cartilage tissue engineering is considered the most promising strategy for the complete regeneration of hyaline cartilage. Unfortunately, optimal seed cell and scaffold material have not yet been found. Therefore, searching for new therapeutic methods with a cartilage regenerative potential is a major goal in cartilage sciences in the future. Limitations and advantages of abovementioned methods should be considered when designing a novel protocol or improvement in the existing procedures.

Cross-References

► Regenerative Medicine Applied to the Treatment of Musculoskeletal Pathologies

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Direct Reprogramming Strategies for the Treatment of Nervous System Injuries and Neurodegenerative Disorders

13

Katarzyna Pieczonka, William Brett McIntyre, Mohamad Khazaei, and Michael G. Fehlings

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Katarzyna Pieczonka and William Brett McIntyre have contributed equally to this chapter.

K. Pieczonka · W. B. McIntyre
Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada

Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada
e-mail: k.pieczonka@mail.utoronto.ca; brett.mcintyre@mail.utoronto.ca

M. Khazaei (✉)
Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada
e-mail: mkhazaei@uhnres.utoronto.ca

M. G. Fehlings (✉)
Division of Genetics and Development, Krembil Research Institute, University Health Network, Toronto, ON, Canada

Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada

Division of Neurosurgery, Department of Surgery, University of Toronto, Toronto, ON, Canada
e-mail: Michael.Fehlings@uhn.ca

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Abstract

Cell transplantation is an attractive therapeutic avenue for injuries of the central nervous system (CNS) and neurodegenerative disorders. Transplanted cells are able to restore the cells that are lost in the injury process, including neurons, oligodendrocytes, and astrocytes. Various clinically relevant cell types and sources have been explored thus far, including induced pluripotent stem cells (iPSCs), which can be used for autologous transplantation. Despite this advantage, differentiation of iPSCs remains time consuming, which may be a limitation in urgent clinical cases. Additionally, the intermediate pluripotent state increases the risk of tumorigenicity when transplanting iPSCs. In this regard, research efforts have shifted toward the transdifferentiation of somatic cells into a variety of neural cell types, including neurons, astrocytes, and oligodendrocytes or their progenitors. This method bypasses the pluripotent stage to reduce the risk of tumorigenicity, thus reducing the induction timeline while still maintaining the patient-specific capacity of the cells. Neural cells or their progenitors can be differentiated in vitro using a number of methods, including transient expression or suppression of certain transcription and chromatin remodeling factors through gene manipulation, or miRNA and small molecule treatment. Recently, research efforts have also focused on in vivo transdifferentiation, in which endogenous cells are targeted for conversion into cell types of interest. The following chapter will focus on the general principles of direct neural lineage conversion, the methods used to derive particular cell types, and their application to injuries of the CNS.

Keywords

Cell source · Cell transplantation · Direct reprogramming · High-throughput screening · Neurodegeneration · Transcription factors · Traumatic CNS injury

Abbreviations

6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
BMA	Ascl1, Brn2, Mytl1
CNS	Central nervous system
CPZ	Cuprizone

CRISPRa	CRISPR activation
DCas9	Dead form of Cas9
DCX	Doublecortin
DRNPCs	Directly reprogrammed neural progenitor cells
HDAC	Histone deacetylase
FACS	Fluorescent activated cell sorting
IPSCs	Induced pluripotent stem cell
INSCs	Induced neural stem cells
miRNA	Micro-RNA
MACS	Magnetic-activated cell sorting
MBD2	Methyl-CpG-binding domain protein 2
MS	Multiple sclerosis
MSI1	Musashi
Ngn2	Neurogenin-1
NSCs	Neural stem/progenitor cells
OPC	Oligodendrocyte precursor cell
ORF	Open reading frame
PD	Parkinson's disease
RA	Retinoic acid
SCI	Spinal cord injury
SCRNASeq	Single-cell RNA sequencing
SGRNA	Single guide RNA
SHH	Sonic hedgehog
TBI	Traumatic brain injury
TF	Transcription factor

Introduction

Initially, it was thought that terminally differentiated cells could not be converted to other cell types from other lineages. However, the pioneering finding that described how fibroblasts could be converted to myoblasts via overexpression of the transcription factor (TF) MyoD demonstrated that different cell lineages could be converted to one another (Davis et al. 1987). This finding has opened a new path for research in developmental biology and regenerative medicine, holding great potential for replacing postmitotic cells lost to disease. Further research has shown that converted cells can bypass the principal developmental stages of differentiation through other routes that are not developmentally defined. This process, called transdifferentiation or direct lineage reprogramming, does not follow developmental differentiation routes and is completed without transitioning through an intermediary pluripotent state. Direct reprogramming can generally be achieved by treating cells with defined cell reprogramming factors (Ibrahim et al. 2016). Therefore, determining the reprogramming factors that can be used for direct reprogramming towards a neural lineage is of high importance.

Throughout this chapter, we will discuss the methods by which cells can be directly reprogrammed to a neural fate. As there is a myriad of synthetic molecules

and gene regulatory elements that could possibly promote a neural fate, screening methods are needed to identify the essential factors that would be able to contribute to high-throughput reprogramming. Importantly, direct reprogramming techniques have been applied in the context of injury by reprogramming the cells in vitro and subsequently transplanting them into animal models, or by promoting endogenous conversion of resident cells in vivo. Nonetheless, considerations related to the cell sources as well as the resulting cellular identity must be addressed to derive a directly reprogrammed cell therapy that can appropriately replace the lost cells.

Direct Neural Reprogramming Strategies

Direct reprogramming of nonneural lineage cells to neural cell types or their progenitors is essential for human applications, as the accessibility to human neural cells is limited. Conventional methods, such as embryonic stem cell and induced pluripotent stem cell (iPSC) differentiation, typically mimic the developmental stages via applying different growth factors or small molecules to cultured cells. However, recapitulating the developmental stages for differentiation of human neural cells is very time consuming because the production of specific human neural cell types takes place over months and years during human brain and spinal cord development. In contrast, the direct reprogramming of fibroblasts or other cell types into neural cells uses alternate, non-developmental routes, ensuring the quick and reproducible generation of these cells that look more like mature neurons or neural subtypes born later. This is completed by screening for different genetic and chemical factors that can convert somatic cells along the neural lineage (Tsunemoto et al. 2015). To identify the minimal and optimal combinations of these factors, different screening methods have been developed. In this section, we discuss these approaches.

Reprogramming Factors

There are three main unique classes of reprogramming factors that have been utilized for the conversion of somatic cells to neural cells: (i) TFs, (ii) microRNAs, and (iii) chemical small molecules. Each of these classes can be used alone or in combination with others.

Transcription Factors

TFs are the key players among the reprogramming factors in lineage conversion. During development, cells become increasingly specialized until they reach a terminally differentiated state, which is mainly controlled by the expression of TFs that form specific expression networks to ensure stable cell identity. In humans, there are ~1500 different TFs, but not all of them are involved in cell, or more specifically, neural differentiation. Thus, only small subsets of TFs that are involved in differentiation are potential candidates to be used for direct reprogramming. However, groundbreaking work from the Wernig lab has shown that the TFs used for lineage

conversion are not necessarily those involved in cell differentiation during normal development (Vierbuchen et al. 2010). Sometimes a not easily predictable combination and sequential expression of TFs can change the lineage of cells from one terminal differentiated identity to another. Some of these TFs are "pioneer TFs," which can bind and open closed chromatin to enable the binding of other canonical TFs that are important for direct neural conversion. For example, Ascl1 is one of the pro-neural pioneer factors that, through ectopic expression, can initialize cells to exit the cell cycle and enter the neuralization route (Vierbuchen et al. 2010). Downstream of Ascl1, Zfp238, Sox8, and Dlx3 are the key canonical TFs that promote the expression of neural-fate specific genes (Wapinski et al. 2017).

Over the past decade, there have been advancements in identifying key TFs that can directly reprogram a somatic cell's fate. TFs can be overexpressed in somatic cells using different delivery methods. Integrating viral approaches (e.g., lentiviral or retroviral) is efficient but risks oncogenic insertion-mutagenesis due to random integration into the genome. Non-integrating viral (e.g., adenoviral or Sendai-virus) or nonviral (e.g. episomal plasmids) approaches have reduced risks but the trade-off results in considerably lower efficiency.

miRNA

miRNAs (or micro-RNAs) are short noncoding RNAs that are mainly demonstrated to mediate downregulation of expression via translational repression or decay of mRNA (Haider et al. 2015). However, miRNAs can indirectly stimulate gene expression. miRNAs have been shown to target the expression of TFs (Shibata et al. 2008) and epigenetic regulators (Gu et al. 2018), the two main contributors in cell fate reprogramming. Viral and nonviral approaches can be used to deliver miRNAs to cells both in vitro and in vivo. Recently, miRNAs received attention as reprogramming factors because of their relatively small size and ability to be administered systemically or locally by nanoparticle-based delivery systems, avoiding the use of viral vectors (Xue et al. 2013). Supporting their functionality for transdifferentiation of neural cells, brain-enriched miRNAs, miR-9/9∗ and miR-124, can convert human fibroblasts into neurons (Yoo et al. 2011). However, miRNAs do not typically function alone, but synergistically with different master TFs. For example, Ambasudhan et al. have reported that miR-124 needs to be supplemented with two TFs, Myt1l and Brn2 to directly reprogram postnatal and adult human primary fibroblasts to functional neurons in vitro (Ambasudhan et al. 2011). In another study, miR-338 has been used to transdifferentiate human stromal cells to pre-oligodendrocytes (Ebrahimi-Barough et al. 2013, p. 338).

Small Molecules

Small molecules are chemical compounds with a low molecular weight that function as agonists or antagonists of proteins and enzymes in signaling pathways or metabolic processes. The small molecules that manipulate signaling pathways controlling cell differentiation (e.g., Wnt, Notch, Smad, or GSK3b pathways) are widely used in approaches to directly reprogram somatic cells (Xie et al. 2017). Also, small

molecules that target epigenetic modifiers have been shown to play an essential role in the transdifferentiation process. Lineage conversion of one cell type to another needs to overcome the epigenetic barriers established during development (Qin et al. 2016). Histone deacetylase (HDAC) inhibitors and DNA methyltransferase inhibitors were among the earliest discovered compounds used for lineage conversion.

Large chemical libraries of compounds (in the order of a million compounds) are currently being used to screen reprogramming factors (Xie et al. 2017). Since small molecule treatment induces cell-lineage reprogramming in a transgene-free manner, the application of small molecules may potentially be easier to translate into therapeutic applications.

Approaches for Determining the Optimal Combination of Reprogramming Factors

Comparative Gene Expression Analyses

One of the approaches to determine the TFs that can be used for direct reprogramming of somatic cells to terminal neural cells is based on systematic comparative genome-wide gene expression analyses between somatic cells and the neural cells of interest or their progenitors, to use TFs that are highly expressed in neural cells compared to somatic cells (D'Alessio et al. 2015). This approach may not be efficient because most of the TFs that exhibit efficacy in direct lineage conversion are not necessarily the most differentially expressed between cell types. Therefore, this method is mainly useful as a starting point to identify potential TFs and is usually combined with alternative methods (discussed below) to determine an ideal cocktail of reprogramming factors for neural conversion.

Trial and Error Screening of Libraries

One alternative approach is random trial and error to screen pooled libraries of differentiation factors. In this approach, the candidate factors are not known a priori. These pools of differentiation factors can be either expression libraries of TFs/miRNAs, chemical small molecules, or a combination of both (Fig. 1). Pools of multiple factors or group of factors are applied to the cells, where one factor or a group of factors is eliminated to find those essential for that specific lineage conversion. However, performing this type of random screening of factors is a slow and tedious process if it is not combined with high-throughput automated readout approaches to identify reprogrammed cells. One of the most common readout approaches is inserting a fluorescent protein reporter under cell type-specific promoters in the initial cells. After applying the library of pooled factors to cells, only the cells that have received the right combination of factors will express the reporter protein.

This strategy enables fluorescence-activated cell sorting (FACS) of cells expressing the fluorescent protein. The common promoters that have been used for cells differentiated to neurons are Tubb3 and Mpa2. For oligodendrocyte and astrocytes, Olig2 and GFAP are the specific promoters that have been used. Deep sequencing approaches employing single-cell RNA sequencing (scRNA-seq) are

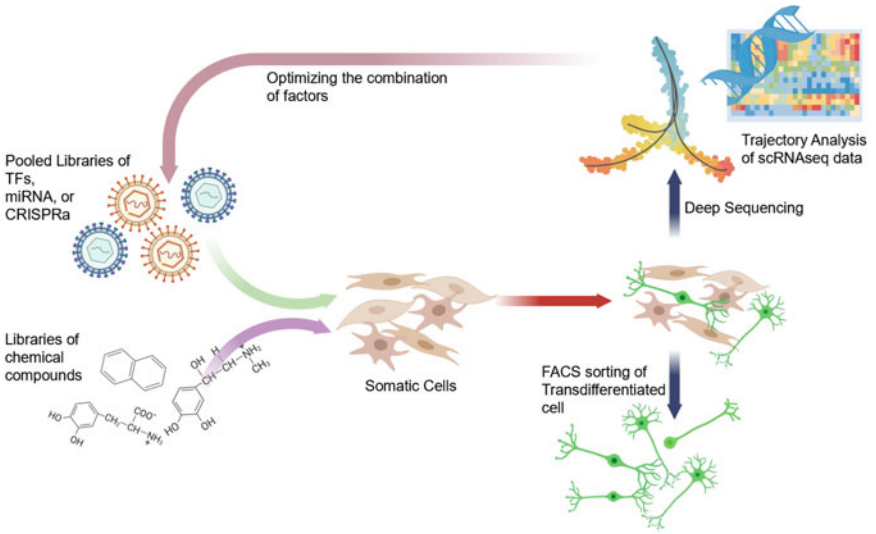

Fig. 1 Determining the optimal combination of reprogramming factors. In most situations, a combination of different approaches are used to screen for the optimal combination of factors to directly reprogram different somatic cells to neural cell types. Genetic screening includes libraries for overexpression of TFs via expressing their open reading frames (ORFs) or activation of their promoters by CRISPRa. Libraries of chemical compounds are used to screen for small molecules. After treating the cells with libraries of reprogramming factors, cells are screened for the target cell phenotype. FACS or deep sequencing are used to identify the factors in genetic screening. Computational approaches are helpful to fine-tune and optimize the combination of identified factors

being used to identify the optimal combination of TFs or miRNAs required to reprogram cells and induce transdifferentiation.

Using this trial and error approach, the Wernig lab identified three TFs: Ascl1, Brn2, and Myt1l (BAM) for direct lineage conversion of mouse fibroblasts to neurons. This was completed within 2–3 weeks, at an efficiency of up to 20% (Vierbuchen et al. 2010). Here, Ascl1 acts as a pioneer factor by immediately occupying most cognate genomic sites in fibroblasts to open the chromatin structure and allows the recruitment of Brn2 and Myt1l to target sites genome-wide. Several other groups have screened for different TF combinations, and notably, almost all successful cocktails contain Ascl (Pfisterer et al. 2011; Yoo et al. 2011; Ambasudhan et al. 2011; Caiazzo et al. 2011). However, the conversion of human fibroblasts to neural cells was more challenging. Developmentally, mouse and human cells have different temporal requirements for neural development involving distinct regulatory strategies. The Wernig lab has found that the addition of NeuroD1 to original BAM factors is necessary to convert human fibroblasts to neurons, but still with lower efficiency (2–4%) than that of mice (20%) (Pang et al. 2011). Several other attempts have been made to improve the transdifferentiation efficiency of human fibroblasts to neurons. In one of these studies, the addition of small molecules to inhibit GSK3β and Smad signaling in the TF cocktail was shown to increase the efficiency to more

than 80% (Ladewig et al. 2012). Later on, Pfisterer et al. identified six small molecules that could increase the neural conversion of human fibroblasts induced by BAM TFs. The six small molecules contain inhibitors of GSK3β and Src kinase pathways, forskolin to activate cAMP-induced pathways, and a balanced combination of an inhibitor and activator of histone deacetylases (Pfisterer et al. 2016).

In a screen to find the optimal factors to convert mouse fibroblasts into neuronal cells, a combination of a priori approaches have been used (Li et al. 2015). In this approach, a library of chemical small molecules is first applied to Ascl1-overexpressing fibroblasts. Ascl1, as a pioneering TF for conversion of somatic cells to neurons, helps to narrow down the screen to small molecules that are only complementary to Asl1. In this screen, forskolin plus three small molecules that inhibit signaling pathways under GSK3β, TGFβ, and noncanonical Wnt were identified. In the next step, researchers sought to determine if Asl1 can also be replaced with small molecules. Ascl1 was omitted and a subsequent small molecule library was screened to replace Ascl1. In this screen, the compound I-BET151 was identified (Li et al. 2015). I-BET151 targets an extensive range of diverse signaling pathways like cyclooxygenase, Aurora kinase, PI3 kinase, and GSK3β pathways. The most probable mechanism for I-BET151 replacing Ascl1 is mediation through affecting the abovementioned pathways.

To convert human fibroblasts into neurons, another screen of chemical compounds identified the inhibitors of MEK and p53 pathways as being required, in addition to forskolin and inhibitors of GSK3β and dual-Smad (TGFβ and BMP) pathways (Dai et al. 2015; Toyokuni 2015).

By comparing different small molecule screens to convert somatic cells to neurons, cAMP activation and GSK3b inhibition (cAMP/GSK3β/sSmad pathway) were deemed canonical for this process. However, depending on the developmental stage of the starting somatic cells, alternative pathways may also need to be targeted. For example, in a screen examining conversion of human adult fibroblasts to neurons, inhibition of TGFβ, PKC, and JNK pathways was found to be required in addition to canonical cAMP/GSK3β/sSmad pathways (Hu et al. 2015). Conversely, conversion of human astrocytes to neurons required activation of retinoid and smoothened pathways in addition to that canonical cocktail (Zhang et al. 2015a).

CRISPR Activation-Based Screening

CRISPR activation (CRISPRa) is a technique that uses the dead form of Cas9 (dCas9) without endonuclease activity fused to transcriptional activators, such as VP64, to induce gene expression. Using this technique, it is possible to activate the expression of any gene of interest by designing a “single guide RNA” (sgRNA) against their promoter regions (Gilbert et al. 2013). Libraries of CRISPRa are popular screening tools due to the relative ease in developing large sgRNA libraries, allowing for potential genome-wide screening. CRISPRa has excellent compatibility with high-throughput screening techniques, making it more attractive for screening of novel TFs involved in direct reprogramming (Fig. 1).

Liu and colleagues of the Qi group at Stanford University have applied the CRISPRa method to screen for TFs that can be used to convert fibroblasts to neurons

(Liu et al. 2018). Specifically, a library of CRISPRa was created against 2428 TFs, which were predicted in silico and applied to mouse fibroblasts. To identify and isolate differentiated neurons, they inserted a human CD8 antigen sequence into the neuronal gene Tubb3. This strategy enabled magnetic-activated cell sorting (MACS) of cells expressing the neuronal marker. Differentiated neurons were isolated by cell sorting and next-generation sequencing was performed to examine which sgRNAs were most efficient for generating neurons. They validated several previously identified TFs, as well as discovered new TFs, such as Ezh2. Ezh2 was identified to promote direct fibroblast to neuron reprogramming in combination with TFs such as Ngn1, Brn2, or Mecom. Furthermore, Ezh2 co-expression with Ngn1 resulted in an increased efficiency of transdifferentiation of fibroblasts to neurons (around 50%) (Liu et al. 2018).

Employing Single-Cell RNAseq and Computational Analysis

Most of the direct reprogramming cocktails discovered thus far for direct reprogramming are generally inefficient, producing incompletely converted and developmentally immature cells that fail to fully recapitulate target cell identity. Incomplete conversion results in heterogeneity among reprogrammed cells during reprogramming. However, the nature of the intermediate states that drive the reprogramming trajectory toward distinct neural types is largely unknown. Understanding the intermediate stages that initial cells undergo to get to the transdifferentiated neuronal fate is key to optimizing the directly reprogramming cocktails. scRNAseq analysis is useful for deconstructing the considerable heterogeneity that emerges during lineage conversion and enables us to distinguish fully converted cells from partially reprogrammed intermediates (Fig. 1). Therefore, cellular reprogramming strategies aimed at generating neural cells have begun to incorporate scRNAseq, alongside computational approaches, to find lineage relationships among intermediate steps that can be used to fine-tune the differentiation processes.

There are several computational frameworks and algorithms that have been developed to predict the combinations of factors that can be used for the transdifferentiation of various cell combinations. For example, Mogrify (http://www.mogrify.net) is a network-based algorithm designed to find TFs crucial for lineage conversion. Mogrify combines gene expression data with regulatory network information to predict the reprogramming factors necessary to induce cell conversion. This algorithm allows for exploring possible reprogramming factors and examining changes in regulatory networks (Rackham et al. 2016).

Implications of the Cell Source

Significant methodological variations exist for the derivation of neural cells depending on the somatic cell source, the germ layer that they are derived from (i.e., mesoderm, ectoderm, endoderm), or the cells' developmental state (Fig. 2). The variant extent of conversion may discredit the use of similar reprogramming factors for different somatic sources. As such, this may hinder a directly reprogrammed

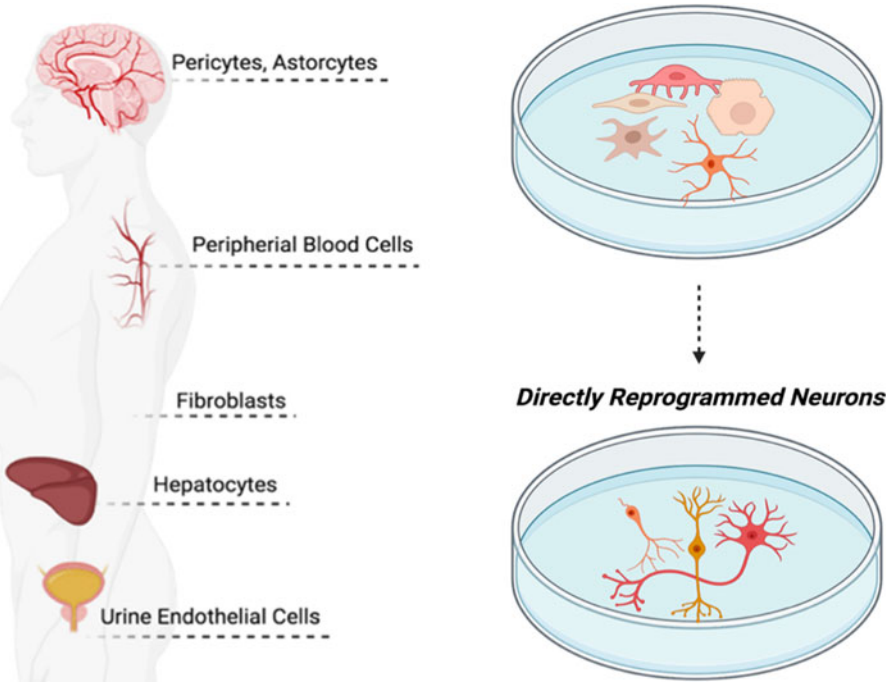

Fig. 2 Schematic of primary-derived cell sources used in the current literature for direct programming

Table 1 Summary of somatic cell sources and their confounding variables in CNS-injury environments if improperly reprogrammed

Somatic source	Germ layer	Potential adverse effects if reprogramming is incomplete
Fibroblast	Ectoderm	Contribute to fibrosis Excrete ECM molecules
Astrocyte	Ectoderm	Possibly become reactive in injury/degenerative environment
Hepatocyte	Endoderm	Secrete albumin
Brain-pericyte	Ectoderm	Contribute to fibrosis by sealing glial scar cavity
Urine tubular epithelial	Mesoderm	Possibly contribute to fibrosis (renal tubular epithelia)

cell's ability to promote an optimal cell replacement therapy. Outlined below are the contexts in which somatic cells are directly reprogrammed towards a neural lineage, as well as implications for their functions if they were not differentiated to a full neural extent (Table 1). This is an important consideration, as the extent of functional conversion in somatic cells is rarely described, making it unclear whether the reprogrammed cells retain unwanted properties from the original cell source. Thus, future work should thoroughly investigate transcriptomic changes, alongside several

other parameters, including but not limited to: functional loss of the original somatic tissues used for reprogramming, electrical activity, their neurotransmitter phenotype, as well as which endogenous targets integrated with the donor neuronal cells in vivo.

Fibroblasts

Fibroblasts are the primary cell type present in the dermis and present vast diversity of structure and function based on their anatomical site of origin. Fibroblasts are frequently used for reprogramming studies as they can be obtained from multiple sources with less invasive procedures. They can be easily expanded in vitro. At site-specific regions, fibroblasts differ in terms of their ability to regulate cell migration, extracellular matrix synthesis and secretion, as well as cell fate specification. The segmentation dictating these regional differences exists on the anterior-posterior (head vs. toe), proximal-distal (torso vs. outer appendages), and internal-external (stroma of organs vs. dermis) axis (Rinn et al. 2008). Thus, fibroblasts are notably heterogeneous and even contain derivatives of the neural crest/neuroectoderm, making them well suited to be converted to functional neural cells. Notably, they are the most widely used source for transdifferentiated and directly reprogrammed cells towards a neural lineage (Marro et al. 2011; Cassady et al. 2014; Tsunemoto et al. 2018; Thier et al. 2019). The extent of conversion towards a neural lineage, however, is crucial when considering their application to the injured brain and spinal cord. For example, the glial scar is present in neural tissue following injury. It comprises dense layers of fibroblasts, infiltrating macrophages, and reactive glial cells that secrete extracellular matrix molecules (Wang et al. 2018). Once transplanted, the functionally incomplete induced neural cells may accumulate and physically impede axonal outgrowth at the glial scar. Moreover, fibroblasts release extracellular matrix molecules, which may further exacerbate the formation of the glial scar. Thus, retention of fibroblast-like structures and functions may impede the regeneration the transplanted cells are attempting to correct.

Astrocytes

Reprogramming of astrocytes towards another neural cell type in vivo presents a potential strategy to overcome the reactive astroglial response following ischemic and traumatic injury. However, the exogenous transplantation of directly reprogrammed astrocytes in vitro would not be clinically relevant because astrocytes are not a readily available cell source, compared to other somatic cells such as fibroblasts. As such, the following discussion refers to astrocyte reprogramming in vivo.

As extensively reviewed elsewhere (Tai et al. 2020), reprogramming of astrocytes or NG2+ astroglia can promote the formation of neurons that are electrophysiological active and promote functional recovery in a variety of trauma and degenerative contexts (Tai et al. 2020). However, the primary issue of in vivo conversion appears

to be the lack of specificity in targetting the desired cells. Remarkably, several studies have evaluated reprogramming efficiency from NG2+ glia (Heinrich et al. 2014, p. 2; Torper et al. 2015; Pereira et al. 2017). However, NG+ glia contains heterogenous cells (astrocytes, oligodendrocytes, and oligodendrocyte precursor cells (OPCs)), which present a variety of somatic cell sources. As such, this results in the mistargeted reprogramming of non-astroglial cells, which leads to different reprogramming efficiencies. Moreover, the heterogeneity of OPCs during trauma further complicates reprogramming efficiencies. Specifically, OPCs rapidly proliferate and contribute to glial scar formation, and provide a new source of astrocytes and oligodendrocytes (Fernandez-Castaneda and Gaultier 2016). This brings into question whether another marker, aside from NG2+ can specifically target reactive astrocytes once they have begun to hyper-proliferate and contribute to glial scar formation.

Although clear benefits can be observed from studies using small molecule treatment (Ma et al. 2021), methods used to induce reprogramming via common TFs (Neurod1, Sox 2, Ascl1) needs further clarification in order to target and reprogram the appropriate cells. This was recently exemplified in a study evaluating the reprogramming effects of Neurod1. Using lineage tracing and a retrograde labelling method, Wang et al. determined that their once presumably reprogrammed astrocytes were actually Neurod1+ expressing endogenous neurons (Wang et al. 2020). Ultimately, this valuable study highlights the need for lineage tracing during in vivo reprogramming of astrocytes to confirm that the conversion of a cell population of interest, and not just overexpression of a particular TF, promotes a therapeutic effect.

Hepatocytes

Hepatocytes are a terminally differentiated cell type of the liver, derived from the endodermal lineage. Directly converted hepatocytes to neurons interestingly exhibit similar reprogramming efficiencies compared to fibroblasts, despite not being derived from the ectodermal developmental lineage. Hepatocytes are another common source of somatic tissue used for direct neural conversion and exhibit comparable differentiation efficiency compared to fibroblasts (Marro et al. 2011). The extent of neural conversion is also a potential issue when applying hepatocytes as a somatic cell source. Interestingly, however, incomplete differentiation of hepatocytes to neural cells may exhibit some utility. Functional neural tissue that exhibits some hepatocyte-like properties (i.e., albumin secretion) may suppress excessive excitotoxicity, a feature of early experimental spinal cord injury (Yildirim et al. 2018). This may peak interest in using hepatocytes as a cell source, as partial retention of function in converted neural cells from hepatocytes may exhibit a neuroprotective strategy. However, global transcriptomic changes following reprogramming are coupled with a loss of hepatocyte function, including albumin secretion and urea production (Marro et al. 2011). Thus, current evidence suggests typical reprogramming strategies are sufficient to restrict the function of hepatocytic

cell sources. The fact that cells that differ greatly from neural tissue can still be converted to the same extent is crucial because this suggests a variety of cell sources can be used for reprogramming. However, hepatocytes are not as easily accessible for exogenous reprogramming, nor do they reside within the CNS. As such, they are not a promising cell source for cell therapies within the CNS.

Pericytes

Pericytes represent an alternative resident cell type of interest within the central nervous system (CNS). In the context of injury states, they proliferate to mediate the intrusion of peripheral leukocytes, where they eventually migrate and contribute to vascularized sealing of the glial scar (Laredo et al. 2019). Moreover, experimentally attenuating their function can promote endogenous corticospinal tract regeneration across the site of injury (Dias et al. 2018). Thus, pericytes represent a potential cell source that can be reprogrammed in vivo following injury. However, the extent of direct pericyte reprogramming to a neural lineage is not commonly described. Pericytes can be reprogrammed into neuronal subtypes, as identified by cholinergic (Liang et al. 2018) and GABAergic (Karow et al. 2012) phenotypes. Although similar programming techniques suggest these cells enter a transient neural stem cell-like fate (Karow et al. 2018), their behavior in an injured transplant environment has yet to be described. Similar to the possible incomplete differentiation of fibroblast cells, their fibrotic/vascularization function in injury contexts may confound their use as an exogenous source of reprogrammed neurons. Moreover, if the extent of conversion is restricted to a neural progenitor-like fate, their differentiation in a transplant environment would likely be astrocytic.

Urine Epithelial Cells

Urine epithelial cells have been used as a cell source for reprogramming because they are a relatively abundant and easily accessible source of autologous tissue. For example, epithelial-like tissue from human urine can be converted to tripotent neural progenitors (Wang et al. 2013). Notably, these cells were synaptically active and expressed a variety of neuronal subtypes markers indicating the presence of GABAergic, glutamatergic, and dopaminergic neurons (Wang et al. 2013, p.; Zhang et al. 2015b). However, there is a heterogeneous population of three major epithelial cells present in urine: renal tubular, transitional, and squamous epithelium (Ringsrud 2001). It remains undescribed in these reprogramming methods whether one epithelial subtype may be more efficiently reprogrammed over another, or if a distinction was made to separate any of these cell types. Notably, incomplete differentiation and retention of epithelial-like characteristics of renal tubular cells could be particularly troubling, as they are prominent contributors to renal fibrosis and rapidly respond and aggravate this pathophysiological process (Qi and Yang 2018).

Generation of Distinct Neural Cell Subtypes

There are several neuronal subtypes present in various brain and spinal cord regions, which relay region-specific functions such as fine motor control, cognition and memory, and even respiration. Even glial function varies significantly between the compartments of the CNS. Thus, direct reprogramming protocols must begin to derive neurons, astrocytes, and oligodendrocytes that can replace distinct cell identities lost due to injury. The developmental origin of a cell is a crucial factor that dictates subtype specification during primary cell derivation (Mothe et al. 2011; Wuttke et al. 2018) and iPSC culturing protocols (Strano et al. 2020). Thus, variations in direct reprogramming methods *between* different somatic tissues (e.g., fibroblast, hepatocyte), as well as *within* a somatic cell type (brain-astrocyte vs. spinal-astrocyte) are likely required to produce the same terminal cell type of interest. Considerations for region-specific differences in these cell types will be discussed below.

Different Subtypes of Astrocytes

Notably, in both the developmental and adult stages, the spinal cord exhibits an enhanced expression of conventional astrocytic markers, such as GFAP and ALDH1L1, compared to whole-brain structures (Yoon et al. 2017). When directly comparing the ability to convert heterogeneous astrocytes derived from a rostral/caudal axis to a neural lineage, reprogramming efficiency was nearly 50% for cortex-derived astrocytes, but only 20% for spinal cord-derived astrocytes (Hu et al. 2019) (visualized in Fig. 3). However, converted astrocytes did not produce different proportions of neuronal subtypes (Hu et al. 2019). This suggests regionally distinct astrocytes are differentially competent to neural conversion but not neuronal subtype specification when using similar reprogramming protocols in vitro. In addition to discrepant reprogramming competency in the context of regional source, in vivo healthy and injury microenvironments present an additional layer of complexity. Notably, the TF *Sox2* can robustly convert various spatially derived astrocytes into induced neurons in vitro (Heinrich et al. 2014; Niu et al. 2015, p. 2), in various injury environments, and the healthy adult striatum (Niu et al. 2013), but cannot exhibit the same competency in the healthy cerebral cortex (Heinrich et al. 2014) (Fig. 4). This highlights critical differences in the intrinsic properties of cells during direct neuronal reprogramming. Future work should highlight the mechanistic variances in reprogramming efficiencies in various brain compartments and different segments of the spinal cord. For example, astrocytes may exhibit functional differences and conversion efficiencies when comparing between a cervical and lumbar-derived astrocyte. Such research can provide a

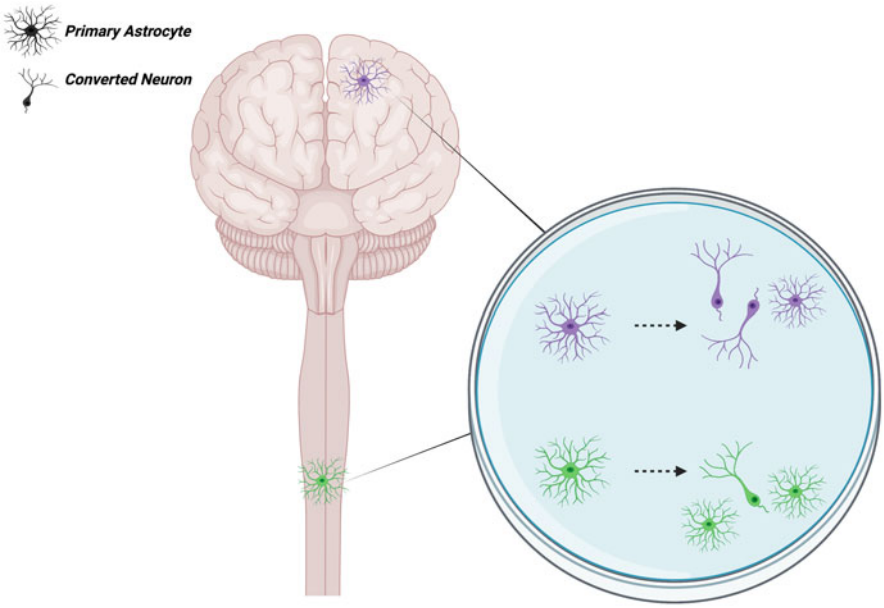

Fig. 3 Visual outline of in vitro reprogramming efficiency of astrocytes to neurons. Regional source of the primary astrocyte may affect reprogramming efficiency in vitro, while using similar reprogramming methods. Variation may include the efficiency or number of neurons produced from regionally distinct astrocyte cultures. Purple = brain-derived cells; green = spinal-derived cells

template for region-specific, directly reprogrammed cellular technologies towards region-specific degeneration/injuries in the CNS.

Different Subtypes of Oligodendrocytes

Regional characterization may be crucial for directly reprogrammed oligodendrocytes, as the brain and spinal cord contain a heterogeneous population of oligodendrocytes with varying functional properties. At a molecular level, there are discrepancies in the endocannabinoid receptor and ligand expression between oligodendrocytes from the brain and spinal cord, highlighting key differences in their migratory and myelination potential (Moreno-Luna et al. 2021). Functionally, spinal-derived oligodendrocytes can form longer myelin sheaths than cortical-derived oligodendrocytes, which mirrors internodal length in vivo (Bechler et al. 2015). Spinal-derived OPCs also exhibit opposite responses compared to forebrain-derived OPCs, in terms of proliferation capacity and response to excitotoxicity (Horiuchi et al. 2017). In response to demyelinating pathophysiology in vivo, the extent of remyelination is more significant in the forebrain

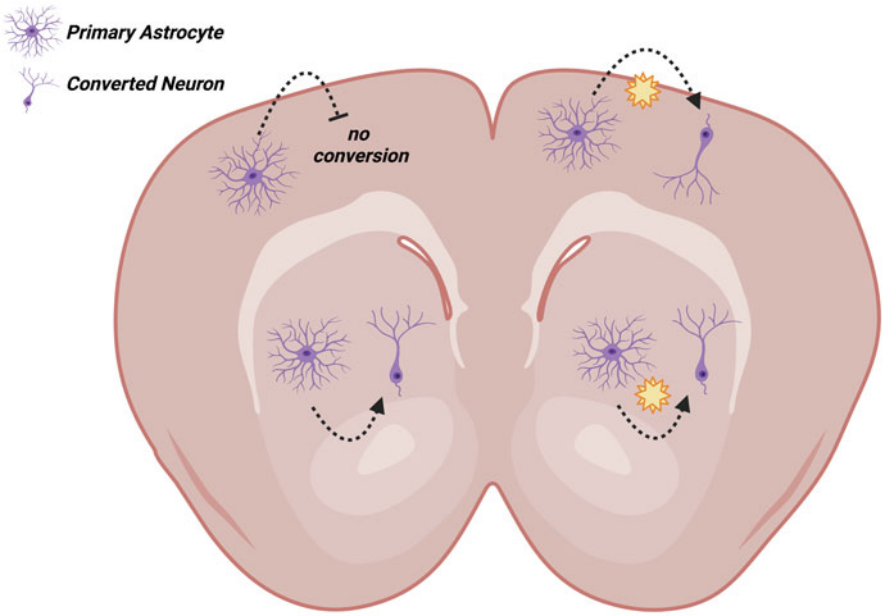

Fig. 4 Visual outline of in vivo reprogramming efficiency of astrocytes to neurons based on spatial region. Similar reprogramming methods in vivo may vary based on spatial region, as well as environmental context. Schematic highlights the necessity of specific-reprogramming paradigms for regional and context-dependent microenvironments (yellow/orange stars indicate injury/degeneration)

compared to the spinal cord (Bradl and Lassmann 2010). These critical functional differences highlight the necessity to derive directly reprogrammed NSCs, or even OPCs that exhibit similar functional properties, to the region they are attempting to restore.

Potential Pitfalls in Autologous Cell Transplantation

Autologous transplants can overcome several ethical and practical issues involving exogenous cell therapy, particularly the enhanced survival of cell grafts due to the reduced risk of host-rejection (Mandai et al. 2017; Song et al. 2020). However, a notable critique of exogenous cell therapy and the use of autologous transplants is the considerable time it takes to culture iPSCs. Once iPSCs have been aptly differentiated into pure NSCs, the therapeutic window in neural injury would have been long passed. Directly reprogrammed neural tissue is an exciting option to circumvent the timely iPSC culturing process. Yet, there are still translational barriers that must be investigated, particularly the age of cell donors.

Directly reprogramming somatic tissues from an older patient population to the neural lineage results in the maintenance of transcriptomic signatures that are age-dependent. These signatures translate to functional losses of cytoplasmic compartmentalization (Mertens et al. 2015) and DNA damage resulting from dysregulated heterochromatin and nuclear organization (Tang et al. 2017). Although these findings promote the use of directly reprogrammed cells for modelling and understanding the pathogenesis of late-onset diseases in culture (Rowe and Daley 2019), it presents several limitations to their use as a source of exogenous transplant therapies. Thus, an "older" autologous cell may retain these aging hallmarks upon direct reprogamming. Moreover, various neurological disorders have some extent of genetic etiology, such as Schizophrenia, Parkinson's disease, and Alzheimer's disease. These disorders, in particular, are often exacerbated with cellular senescence. In the rare case that a patient may be genetically predisposed to a neurological disorder, their genetic abnormalities may be translated into reprogrammed autologous cell grafts. Thus, neural cells prone to dysfunction and death could further confound the state of injury when applied as a transplant therapy. In theory, age and genetic predisposition may impair a directly reprogrammed cells' function. Still, the extent of dysfunction that an "older" reprogrammed cell may present when applied as a therapy requires further experimental investigation.

Treating CNS Disorders Using Transdifferentiated Neural Cells

Despite the advantages that direct reprogramming strategies present over other cellular methods, it is crucial to confirm that the cells contribute to meaningful therapeutic benefits in the context of disease. These cell therapies aim to address CNS pathologies by targeting a wide range of pathophysiological processes including inflammation, demyelination, neuronal disruption, and gliosis. Ultimately, their broad applications make them useful in a number of CNS disorders, which will be described in the following section and are summarized in Table 2.

Spinal Cord Injury

Spinal cord injury (SCI) involves a primary injury caused by mechanical trauma, which induces neural cell disruption, ionic imbalance, and vascular changes. As the injury progresses, the secondary injury ensues, further contributing to neuronal death, demyelination, ionic imbalance, excitotoxicity, and the formation of a glial scar in the chronic phases of SCI (Alizadeh et al. 2019). Various cell sources have been used for the derivation of neural cells that can be transplanted to target SCI. Human adipose-derived stem cells have been successfully directly reprogrammed into neuron/motor neuron-like cells via small molecule treatment with retinoic acid (RA), sonic hedgehog (Shh), and neurotrophic factors. Upon transplantation into injured mice, these cells synaptically integrated and facilitated motor improvements.

Table 2 Summary of direct reprogramming strategies that have been investigated in the context of CNS injuries and disorders

Spinal cord injury			
Source	**Method**	**Final cell type**	**Functional outcome**
Human adipose-derived stem cells (Gao et al. 2019)	• In vitro reprogramming • Small molecule treatment with RA, Shh, and neurotrophic factors	Neuron/motor neuron-like cells	• Motor improvements
Rat mesenchymal stem cells(Qiu et al. 2015)	• In vitro reprogramming • Adenoviral transfection with NT-3 and Trk-C	Neural-like cells and myelin-forming cells	• Motor improvements
Human bone marrow somatic cells (Nagoshi et al. 2018)	• In vitro reprogramming • Transfection with Msi1, Ngn2, and MBD2 • Small molecule treatment with RA, Shh, PDGF, and thyroxine	Oligodendrogenically biased neural progenitor cells	• Motor improvements
Endogenous astrocytes (Su et al. 2014)	• In vivo reprogramming • Lentiviral transfection with SOX2 under the GFAP promoter	Neurons	• Behavioral outcomes were not assessed
Traumatic brain injury			
Source	**Method**	**Final cell type**	**Functional outcome**
Endogenous glia (astroglial conversion was not observed) (Heinrich et al. 2014)	• In vivo reprogramming • Retroviral transfection with Ascl1 and Sox2	Neurons	• Behavioral outcomes were not assessed
Endogenous glia (Guo et al. 2014)	• In vivo reprogramming • Retroviral transfection with NeuroD1 under the GFAP promoter	Neurons	• Behavioral outcomes were not assessed
Ischemic stroke			
Source	**Method**	**Final cell type**	**Functional outcome**
Human bone marrow somatic cells (Vonderwalde et al. 2020)	• In vitro reprogramming • Transfection with Msi1, Ngn2, and MBD2	Neural progenitor cells	• Sensorimotor improvements
Endogenous astrocytes (Chen et al. 2020)	• In vivo reprogramming • Adenoviral transfection with NeuroD1 under the GFAP promoter	Neurons	• Motor and cognitive improvements
Multiple sclerosis			
Source	**Method**	**Final cell type**	**Functional outcome**
Human bone marrow somatic cells, fibroblasts, and keratinocytes (Ahlfors et al. 2019)	• In vitro reprogramming • Transfection with Msi1, Ngn2, and MBD2	Neural progenitor cells	• Behavioral outcomes were not assessed

(continued)

Table 2 (continued)

Spinal cord injury			
Mouse embryonic fibroblasts (Sullivan et al. 2020)	• In vitro reprogramming • Lentiviral transfection with Klf4, Sox2, c-Myc, and Oct4	Neural stem cells	• Motor improvements
Human bone marrow-derived mesenchymal stem cells (Ben-Zwi et al. 2019)	• Small molecule treatment using a patented method	Neuralized mesenchymal stem cells and neurons	• Motor improvements
Endogenous mouse astrocytes (Ghasemi-Kasman et al. 2018)	• In vivo reprogramming • Lentiviral transfection with miR-302/367 • Small molecule treatment with valproate	Oligodendrocyte progenitor cells and oligodendrocytes	• Cognitive improvements
Parkinson's disease			
Source	**Method**	**Final cell type**	**Functional outcome**
Human spermatogonial stem cells (Yang et al. 2019)	• In vitro reprogramming • Small molecule treatment with a series of factors	Dopaminergic neurons	• Motor improvements
Fetal liver mesenchymal stromal-like cells (Kumar et al. 2016)	• In vitro reprogramming • Small molecule treatment with B27, vitamin c, FGF2, Shh, FGF8, TGFβ3, BDNF, and GDNF	Dopaminergic neuron-like cells	• Motor improvements
Mouse embryonic fibroblasts (Dell'Anno et al. 2014)	• In vitro reprogramming • Lentiviral transfection with Ascl1, Nurr1, and Lmx1a	Dopaminergic neurons	• Motor improvements
Mouse Sertoli cells (Wu et al. 2015)	• In vitro reprogramming • Retroviral transfection with Ascl1, Ngn2, Hes1, Id1, Pax6, Brn2, Sox2, c-Myc, and Klf4 followed by lentiviral transfection with Lmx1a	Dopaminergic neurons	• Motor improvements
Endogenous mouse astrocytes (Rivetti di Val Cervo et al. 2017)	• In vivo reprogramming • Lentiviral transfection with NeuroD1, Ascl1, Lmx1a, and miR218 under the GFAP promoter	Dopaminergic neurons	• Motor improvements
Alzheimer's disease			
Source	**Method**	**Final cell type**	**Functional outcome**
Endogenous mouse astrocytes (Guo et al. 2014)	• In vivo reprogramming • Retroviral transfection with NeuroD1 under the GFAP promoter	Neurons	• Behavioral outcomes were not assessed

Additionally, the cells also reduced the inflammatory response, as indicated by increased levels of anti-inflammatory markers (Gao et al. 2019). Comparably, mesenchymal stem cells have also been used as a source of neural-like cells through genetic modification with NT-3 and TrkC. After transplantation, these cells acquired a myelin-forming phenotype and improved cortical motor evoked potential and hind-limb locomotion (Qiu et al. 2015). Moreover, human bone marrow somatic cells have been used for the generation of neural progenitor cells, which were further biased into an oligodendrogenic fate. Briefly, the somatic cells were transiently transfected with Musashi-1 (Msi1), Neurogenin-2 (Ngn2), and methyl-CpG-binding domain protein 2 (MBD2), and subsequently treated with RA, SHH agonist, PDGF, and thyroxine in vitro. When transplanted, these cells contributed to remyelination and axonal sparing, which correlated to improved motor function (Nagoshi et al. 2018). As such, in vitro reprogramming methods and subsequent cell transplantation have proven to be a promising therapeutic strategy for SCI. However, the integration of these cells is often limited due to the glial scar that forms surrounding the lesion. As such, since SCI is followed by reactive astrogliosis, a potentially useful application of direct reprogramming techniques is for endogenous cells to be used as a source to generate novel neurons, thereby indirectly reducing the number of scar-forming reactive astrocytes that surround the lesion. In this regard, in vivo reprogramming is limited in SCI; however, SOX2-mediated conversion of astrocytes to neurons has been reported. This was achieved by delivering a lentivirus that expressed SOX2 under the astrocyte GFAP promoter immediately after a hemisection injury. Notably, the infected cells began expressing neuronal markers, TUBB3 and MAP2, by 4 and 5 weeks post-injury, respectively. The infected cells also co-stained for the synapse marker, synapsin-1, suggesting that they could form synapses. Although no functional outcomes were tested in this paper, neuronal differentiation and synapse formation suggest circuit-specific functions can be recovered. Thus, future studies investigating the causal effects of vivo conversion of resident astroglial on functional recovery are warranted (Su et al. 2014).

Traumatic Brain Injury

Traumatic brain injury (TBI) is followed by a series of maladaptive processes, including immune cell infiltration, inflammation, excitotoxicity, vascular disruption, neuronal death, myelin disruption, and reactive gliosis (McGinn and Povlishock 2016). Animal models of TBI have demonstrated that the targeted delivery of pro-neuronal genes to endogenous glial cells facilitates their conversion into neurons. In one study, a retrovirus containing Ascl1, or a combination of Ascl1 and Sox2, was delivered following a stab wound injury in the upper layers of the cerebral cortex in mice. These constructs facilitated the reprogramming of NG2+ glia along the neuronal lineage, as marked by the immature neuron marker, doublecortin (DCX). Over time, these cells matured into NeuN+ neurons. Most importantly, electrophysiological techniques demonstrated that these cells were forming synapses with the surrounding cells, demonstrating that in vivo direct reprogramming

methods can generate functional neurons (Heinrich et al. 2014). However, it is important to acknowledge that the retroviral delivery method targets dividing cells, which involve astroglial and oligodendroglial cells. Yet, gliosis can be an adaptive process through the generation of novel oligodendrocytes, which can contribute to remyelination. Therefore, the reprogramming of these oligodendroglial cells may be counterproductive in that it may impede subsequent endogenous remyelination efforts by reducing the numbers of myelinating oligodendrocytes. Instead, it may be more helpful and prudent to focus on reprogramming the maladaptive astroglial cells that surround the injury site and limit regeneration.

Nonetheless, evidence demonstrating that astrocytes can be reprogrammed into neurons in the context of TBI has also been reported. In this study, a retrovirus delivery method was used to deliver NeuroD1, a basic helix loop helix TF involved in neurogenesis, into the mouse somatosensory cortex following a stab wound injury. This report demonstrated the conversion of the resident glial GFAP+ and NG2+ cells into novel DCX+ and NeuN+ neurons. Importantly, it was found that astrocytes were the source of newly generated glutamatergic neurons, which were electrophysiologically active (Guo et al. 2014). Therefore, the novel population of dividing glial cells provides a cell source that can be converted into functional neurons. However, future studies should aim to determine the behavioral changes that are associated with these strategies.

Ischemic Stroke

Ischemic stroke occurs when blood flow to the brain is impaired, thus reducing oxygen supply at the site of injury. Like TBI and SCI, this process is followed by several pathophysiological processes including excitotoxicity, inflammation, oxidative stress, and gliosis, all of which contribute to neuronal death and circuit disruption (Kuriakose and Xiao 2020). Therefore, the generation of new neurons is a plausible method by which novel circuitry can be established. Notably, transplantation of directly reprogrammed cells has shown promise in a endothelin-1 mouse model of focal ischemic stroke. Before transplantation, human bone marrow cells were converted into directly reprogrammed neural progenitor cells (drNPCs) by introducing Msi1, Ngn2, and MBD2. Four days after injury, reprogrammed drNPCs were transplanted into the lesion site. Importantly, behavioral tests, including both the foot fault task (assesses sensory and motor function) and the cylinder test (assesses locomotor function), revealed that drNPC transplantation was associated with improved functional outcomes. The authors hypothesized that this recovery was mediated through synaptic plasticity, as immunostaining revealed that drNPC transplantation was associated with an increase in synaptophysin expression (Vonderwalde et al. 2020). In addition, in vivo direct reprogramming has been investigated in the context of ischemic stroke. In this report, an adeno-associated viral construct carrying the pro-neurogenic gene NeuroD1 under the GFAP promoter was injected into the lesion site 10 days following ET-1-induced ischemia. This method facilitated the conversion of reactive astrocytes to electrophysiologically

functional neurons. Importantly, this astrocyte-to-neuron conversion was associated with improvements in motor deficits as assessed using food pellet retrieval, grid walk, and cylinder tests. Cognitive improvements were also observed in these animals, marked by improved fear conditioning memory (Chen et al. 2020). Overall, these preliminary reports demonstrate that directly reprogrammed cells can contribute to meaningful functional benefits following ischemic stroke.

Multiple Sclerosis

Multiple sclerosis (MS) is a neurodegenerative condition in which the immune system targets myelin for degradation. This results in axonal damage to the demyelinated neurons (Faissner et al. 2019). Several reports have described the transplantation of transdifferentiated cells into MS animal models to replenish the lost neurons and myelinating oligodendrocytes. Notably, bone-marrow cells, human fibroblasts and keratinocytes have been used for drNPC reprogramming by using Msi1, Ngn2, and MBD2. Upon transplantation into shiverer mice, which lack compact myelin, drNPCs have been shown to differentiate into oligodendrocytes (Ahlfors et al. 2019). Nonetheless, further analysis of these cells should assess functional improvements. In this regard, several other reports have assessed functional outcomes in MS animal models. For example, mouse embryonic fibroblasts were converted to induced NSCs through the continuous expression of Klf4, Sox2, and c-Myc, combined with the early temporal expression of Oct4. Upon transplantation into the corpus callosum of mice treated with cuprizone (CPZ), an agent that induces demyelination, these cells were associated with motor improvements as demonstrated using running misstep wheels. Moreover, there was an increase in oligodendrogenesis, showing that this is a plausible strategy for oligodendrocyte replacement (Sullivan et al. 2020). While these cell transplantation methods aim to address the neural deficits seen in MS, they have minimal affect on inflammatory-associated pathology. Notably, Ben-Zwi et al. generated neuralized MSCs, which were uniquely suited to address the neural deficits in conjunction with the inflammatory response. These cells contributed to myelin and neuronal sparing, as well as reduced inflammation following transplantation into mice with experimental autoimmune encephalomyelitis – a model of MS – in comparison to non-neuralized control MSCs. More importantly, the neuralized MSCs promoted motor function (Ben-Zwi et al. 2019). As such, transplantation of directly reprogrammed cells has been shown to contribute to functional benefits in MS. In vivo reprogramming has also been shown to promote recovery in the context of MS. Specifically; miR-302/367 and valproate treatment has been shown to convert endogenous astrocytes to OPCs and oligodendrocytes in CPZ-treated mice. Behavioral testing revealed that this in vivo conversion correlated with improvements in cognitive function (Ghasemi-Kasman et al. 2018). Overall, the evidence shows that both in vitro and in vivo cell reprogramming techniques can help generate new cells capable of promoting behavioral and cognitive benefits in MS.

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder marked by an accumulation of Lewy bodies, comprised of α-synuclein, which contributes to the progressive loss of dopaminergic neurons in the substantia nigra (Simon et al. 2020). Several reports have directly converted non-neuronal cells into dopaminergic neurons for transplantation into PD models. One paper reported the conversion of human spermatogonial stem cells into dopaminergic neurons by small molecule treatment in vitro. When transplanted into the MPTP PD model in mice, these cells contributed to synapse formation, electrophysiological activity, and most importantly, they facilitated improvements in gait (Yang et al. 2019). In another report, fetal liver mesenchymal stromal-like cells were converted into dopaminergic neurons through chemical treatment with neurotrophic factors. Upon transplantation into the 6-hydroxydopamine (6-OHDA)-lesioned striatum, these cells contributed to enhanced behavior compared to non-converted MSC control cells in mice 2 months post-transplantation (Kumar et al. 2016).

Moreover, mouse embryonic fibroblasts have also been used as a source of cells for conversion into dopaminergic neurons. These cells were also capable of contributing to electrical activity, synaptic activity, and ultimately promoted functions in a PD model (Dell'Anno et al. 2014). Another report converted Sertoli cells into induced NSCs (iNSCs) and further biased them toward the dopaminergic neuron lineage through the forced expression of Lmx1a. These biased cells exhibited increased motor recovery upon transplantation into mouse PD models (Wu et al. 2015). This shows that a plethora of studies that aim to generate functional dopaminergic neurons for transplantation into PD exist. However, in vivo neuronal conversion is less described in the context of PD. In a single paper, the combination of three TFs, NeuroD1, Ascl1, and Lmx1a, as well as the microRNA miR218, was used to target endogenous mouse astrocytes into dopaminergic neurons. Importantly, this astrocyte-to-neuron conversion was associated with an improvement in gait (Rivetti di Val Cervo et al. 2017). Ultimately, directly reprogrammed dopaminergic neurons show promise in ameliorating PD.

Alzheimer's Disease

Alzheimer's disease is characterized by an accumulation of amyloid-β plaques and neurofibrillary tangles, which contribute to neurodegeneration. Moreover, it involves the death of cholinergic neurons in the forebrain (Hampel et al. 2018). As such, neuronal replacement is thought to be one method that could promote the regeneration of this condition. A single paper has utilized direct conversion techniques in this disease, whereby a retroviral construct expressing NeuroD1 under the GFAP promoter was used to infect astrocytes in vivo in an experimental transgenic mouse model of AD. This facilitated the conversion of astrocytes into NeuN+ neurons, which formed glutamatergic synapses. Notably, the cells were electrophysiologically active. However, further analysis of the potential

therapeutic benefits is warranted to determine whether in vivo reprogramming can drive cognitive benefits (Guo et al. 2014).

Moreover, it is important to acknowledge that cholinergic cells are implicated in processes such as memory and learning (Hampel et al. 2018). Therefore, optimal cognitive recovery will likely rely on the replacement of this particular subtype of neurons. As such, future research should focus on the cholinergic specification of reprogrammed neurons.

Conclusions

Cell replacement is an important therapeutic strategy in a variety of CNS disorders. This can be facilitated through direct reprogramming technologies, which generate neural cells both in vitro and in vivo. Direct reprogramming of somatic cells into the desired cells without the intermediate pluripotent state remains a less explored strategy but would be a major step toward the generation of cells without tumorigenic potential (Ahmed et al. 2012). To date, several reprogramming strategies have been described, including the use of TFs, microRNAs, and the TF-free use of small molecules for direct reprogramming (Ullah et al. 2021). Nonetheless, several questions remain surrounding the ideal cell source and resulting cell identity (Heng et al. 2005). Future investigations will be needed to optimize these reprogramming techniques.

Cross-References

- ▶ Advances, Opportunities, and Challenges in Stem Cell-Based Therapy
- ▶ Common Ethical Considerations of Human-Induced Pluripotent Stem Cell Research
- ▶ Glial Cells in Neuroinflammation in Various Disease States
- ▶ Induced Pluripotent Stem Cells
- ▶ Neural Stem Cells
- ▶ The Potential of Stem Cells in Ocular Treatments

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Therapeutic Effects of Mesenchymal Stem Cells on Cognitive Deficits

14

Carl Randall Harrell, Ana Volarevic, and Vladislav Volarevic

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Abstract

Due to their multilineage differentiation capability and their potent immunosuppressive and proangiogenic characteristics, mesenchymal stem cells (MSCs) have been considered as new therapeutic agents for the treatment of inflammatory and degenerative diseases of the central nervous system. In vitro, MSCs may differentiate into neuron-like cells. In vivo, MSCs, in a juxtacrine and paracrine manner, suppress detrimental immune responses in the brain and spinal cord, attenuate

C. R. Harrell
Regenerative Processing Plant, LLC, Palm Harbor, FL, USA
e-mail: dr.harrell@regenerativeprocessingplant.org

A. Volarevic
Department of Cognitive Psychology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
e-mail: ana.volarevic@medf.kg.ac.rs

V. Volarevic (✉)
Department of Genetics and Department of Microbiology and immunology, Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

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neuroinflammation, and promote the repair and regeneration of injured neural tissue. MSCs produce a large number of trophic and growth factors, induce neovascularization, and prevent apoptotic loss of injured neural cells. Alzheimer's disease (AD) is a neurodegenerative disease that is incurable and characterized by progressive cognitive dysfunction and memory loss. Since the incidence of AD has been continuously increasing in the past decade, new therapeutic agents for the treatment of AD are urgently needed. Several recently published experimental studies revealed that MSCs, through the delivery of neuroprotective and immunomodulatory microRNAs (miRNA), neural growth factors, and anti-inflammatory cytokines, significantly reduced neuronal loss, increased neurogenesis and synaptogenesis, and efficiently improved cognitive impairment. In this chapter, we summarized current knowledge about molecular and cellular mechanisms that were responsible for the MSC-based improvement of cognitive function in experimental animals, and we emphasized issues that should be addressed before MSCs and their secretome could be offered as a new human remedy for the treatment of AD.

Keywords

Alzheimer's disease · Cognitive impairment · Mesenchymal stem cells · Therapy

Abbreviations

AD	Alzheimer's disease
AF	Amniotic fluid
AF-MSCs	Amniotic-fluid-derived mesenchymal stem cells
ALS	Amyotrophic lateral sclerosis
ASDs	Autism spectrum disorders
AT	Adipose tissue
BACE1	β-site amyloid precursor protein cleaving enzyme 1
BDGF	Brain-derived growth factor
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MSCs	Bone-marrow-derived MSCs
CB	Cord blood
CD	Cluster of differentiation
CDNF	Cerebral dopamine neurotrophic factor
CTLs	Cytotoxic CD8 + T cells
CXCR4	Chemokine receptor
DCs	Dendritic cells
d-MAPPS	Derived Multiple Allogeneic Proteins Paracrine Signaling
DP	Dental pulp
EGF	Epidermal growth factor
FGF-21	Fibroblast growth factor 21
HD	Huntington's disease
HGF	Hepatocyte growth factor
HO-1	Hemeoxygenase-1
IDO	MSC-derived indolamine 2,3-dioxygenase

IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK1	Interleukin-1-receptor-associated kinase
ISCT	International Society for Cellular Therapy
MCP	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
miRNA	MicroRNA
MSC-Exos	Mesenchymal-stem cell-derived exosomes
MSCs	Mesenchymal stem cells
MWM	Morris water maze
NeuN	Neuronal nuclear protein
NK	Natural killer
NKT	Natural killer T
NO	Nitric oxide
NOR	Novel object recognition
NSC	Neural stem cell
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PFC	Prefrontal cortex
PGE2	Prostaglandin E2
PGF	Placental growth factor
PL	Placenta
PL-MSCs	Placenta-derived MSCs
RORγT	Retinoic-acid-receptor-related orphan receptor gamma T
RVG	Neurotropic rabies virus glycoprotein
S1K	Sphingosine kinase
S1P	Sphingosine-1-phosphate
SSEA-3	Stage-specific embryonic antigen 3
SSEA-4	Stage-specific embryonic antigen 4
STAT	Signal transducer and activator of transcription
T-bet	T-box protein expressed in T cells
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRAF6	TNF-receptor-associated factor 6
UC	Umbilical cord
UC-MSCs	Umbilical-cord-derived mesenchymal stem cells
VEGF	Vascular endothelial growth factor

Introduction

Neurocognitive disorders belong to the group of organic brain syndrome caused by neurodegenerative processes, which lead to impaired cognitive functioning, memory problems, difficulties in the perception of language, and various behavioral changes (He et al. 2021). Neurodegenerative processes also lead to neuronal deterioration and

gradual loss of cognitive and neurological functions (Lilamand et al. 2020). The type of disorder and its cause determine, in the first place, which age group will be affected. Diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) usually affect the elderly population. However, drug and alcohol abuse can cause these processes in the younger population as well (Luck et al. 2017). Vitamin deficiency, meningitis, encephalitis, and traumatic brain injuries cause neurocognitive dysfunction independent of age. Cardiovascular problems, diabetes, alcohol, and drug abuse significantly increase the possibility of neurological dysfunction and are, therefore, considered risk factors for neurocognitive diseases (Khan et al. 2021).

Cognitive and behavioral dysfunctions, manifested by difficulties in thinking, remembering, and reasoning, are all symptoms of neurocognitive organic syndromes, which develop as a consequence of gradual neuronal loss and degeneration (He et al. 2021). Additionally, all neurocognitive disorders may be accompanied by other symptoms, such as anxiety, headaches, vision loss, attention problems, and difficulties in performing routine tasks. The long-term prognosis of neurocognitive diseases remains challenging and uncertain since a definite cure for these conditions does not exist and the symptoms tend to aggravate and worsen over time (He et al. 2021).

Neurodegeneration and neuroinflammation are the leading causes of neurocognitive dysfunction (Subramaniyan and Terrando 2019; Süβ et al. 2021; Hook et al. 2020). Progressive atrophy accompanied by the loss of neural cell function is observed in specified regions of the brains of patients suffering from neurodegenerative diseases (amyotrophic lateral sclerosis (ALS), PD, AD, HD, prion diseases) (Hook et al. 2020). Metabolic dysfunction and alteration in cell-death-related signaling pathways in neural cells is the main reason for global developmental delay, expressive language delay, and intellectual disability in early childhood (Hook et al. 2020). Later on, in adolescence or early adulthood, due to progressive neurological regression, these patients continue to deteriorate with worsening neurological symptoms and a significant cognitive function decline (Hook et al. 2020). Bradykinesia, rigidity, loss of expressive language skills, epilepsy, and dysfunctional sleep are frequently part of the clinical picture observed in patients suffering from neurodegenerative diseases, affecting their quality of life and functionality (Subramaniyan and Terrando 2019; Süβ et al. 2021; Hook et al. 2020).

In addition to neurodegeneration, chronic inflammation is the hallmark of neurocognitive diseases (Walker 2019) and is considered the main reason for the progressive and irreversible loss of neural cells (Süβ et al. 2021). Responding to microbial infections, innate immune cells (macrophages, dendritic cells (DCs), natural killer (NK) and natural killer T (NKT) cells) generate a variety of pro-inflammatory cytokines and chemokines that increase the permeability of the blood-brain barrier (Subramaniyan and Terrando 2019). Increased production of proinflammatory cytokines (interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α)) and chemokines induce enhanced expression of E and P selectins on endothelial cells, enabling the recruitment of circulating leukocytes in the inflamed brain region (Subramaniyan and Terrando 2019; Süβ et al. 2021). Recruited DCs and

macrophages capture and phagocyte microbes degrade microbial antigens and form small polypeptide fragments, which are, within major histocompatibility complex (MHC) molecules, presented to the naïve or effector T cells, respectively (Subramaniyan and Terrando 2019). Th1 and Th17 cell-driven immune responses are mainly responsible for the development of inflammation-induced loss of neural cells (Holley and Kielian 2012). Activated, proinflammatory DCs deliver microbial antigens to the regional lymph nodes and activate antigen-specific naïve CD4+ and CD8 + T cells, resulting in the generation of T-cell-dependent cellular immune response (Subramaniyan and Terrando 2019; Süβ et al. 2021). Driven by DC-derived cytokines, naïve CD4 + T cells differentiate into effector, inflammatory CD4 + Th1, and Th17 helper cells (Süβ et al. 2021). DC-derived IL-12 and NK cell-sourced interferon gamma (IFN-γ) activate T-box protein expressed in T cells (T-bet) and signal transducer and activator of transcription (STAT)-4 transcriptional factors in naïve T cells, resulting in the generation of CD4+ and CD8+ Th1 lymphocytes (Stojić-Vukanić et al. 2020). DC-sourced IL-1β, IL-6, IL-23, and TGF-β induce the generation and expansion of Th17 CD4+ and CD8+ T lymphocytes through the activation of retinoic-acid-receptor-related orphan receptor gamma T (RORγT) and STAT-3 transcriptional factors in naïve T cells (Süβ et al. 2021; Hook et al. 2020; Stojić-Vukanić et al. 2020).

Within the inflamed tissue, CD4 + Th1 and Th17 cells activate phagocytes and enhance their proinflammatory properties (Harry 2021). CD4 + Th1 cell-sourced IFN-γ activates inflammatory M1 macrophages and M1 microglia, while CD4 + Th17 cells produce IL-17 and IL-22, which activate inflammatory N1 neutrophils to produce reactive oxygen species and inflammatory cytokines (TNF-α, IL-1β), crucially contributing to the aggravation of ongoing inflammation (Süβ et al. 2021; Hook et al. 2020; Stojić-Vukanić et al. 2020).

In addition to innate immune cells, effector CD4 + Th1 and Th17 cells orchestrate antigen-specific antimicrobial immune responses as well (Stojić-Vukanić et al. 2020). Antigen-presenting DCs activate naïve CD8 + T cells, which proliferate and differentiate into effector, cytotoxic CD8 + T cells (CTLs) under the influence of CD4 + Th cell-derived IL-2 (Sallusto and Lanzavecchia 2002). At the same time, cross-talk between effector CD4 + Th cells and B cells is crucially important for optimal humoral immune response (Subramaniyan and Terrando 2019; Süβ et al. 2021). IFN-γ, derived from CD4 + Th1 cells, induces Immunoglobulin (Ig) M and IgG antibody class switching and promotes synthesis and the production of antigen-specific IgG antibodies, which in turn regulate the antibody-dependent activation of macrophages, microglia, NK cells, and complement systems (Hook et al. 2020; Stojić-Vukanić et al. 2020; Harry 2021). This "inflammatory loop" and cooperation between innate and acquired immunity crucially contribute to the efficient elimination of foreign pathogens in the central nervous system (Harry 2021). However, the uncontrolled activation of immune cells may result in the development of chronic inflammation in the brain, which leads to the irreversible injury of neural cells (Süβ et al. 2021; Hook et al. 2020; Stojić-Vukanić et al. 2020; Harry 2021).

In order to prevent excessive tissue injury, immunosuppressive immune cells (alternatively activated macrophages, tolerogenic DCs, and regulatory Treg cells)

produce neurotrophins and anti-inflammatory cytokines (IL-10, IL-35, and transforming growth factor beta (TGF-β)), which attenuate ongoing inflammation; provide neuroprotection; stimulate axonal regeneration; and maintain homeostasis in the central nervous system (Subramaniyan and Terrando 2019). Since a weakened immune response is incapable of eliminating pathogenic microorganisms while an excessive immune response aggravates neuroinflammation and neurodegeneration, a balance between proinflammatory and anti-inflammatory immune cells has to be achieved for the optimal treatment of neurocognitive disorders (Kim et al. 2019). The most effective anti-inflammatory and neuroprotective therapeutic agents should prevent progressive loss of neural cells, induce axonal regeneration, stimulate an antimicrobial immune response, and promote the generation and expansion of immunosuppressive cells in the central nervous system (He et al. 2021). Correspondingly, therapeutic agents, which are able to suppress a harmful immune response and simultaneously provide trophic support to injured neurons, could be considered new remedies for the treatment of neurocognitive diseases (He et al. 2021).

Results obtained in recently conducted experimental and clinical studies demonstrated the therapeutic potential of mesenchymal stem cells (MSCs) in the treatment of neurodegenerative, neuroinflammatory, and neurocognitive diseases (Castorina et al. 2015; Harrell et al. 2021b; Andrzejewska et al. 2021). In this chapter, we emphasized current knowledge about molecular and cellular mechanisms, which are responsible for the beneficial effects of MSCs in the therapy of neurocognitive disorders.

MSCs and Their Exosomes as New Therapeutic Agents in Regenerative Neurology and Neuroimmunology

MSCs are adult stem cells which promote tissue repair and regeneration of postnatal tissues (Shariati et al. 2020). MSCs could be derived from many adult as well as fetus-associated tissues, including bone marrow (BM), adipose tissue (AT), umbilical cord (UC), cord blood (CB), amniotic fluid (AF), dental pulp (DP), placenta, synovia, and peripheral blood (Pittenger et al. 2019; Gazdic et al. 2015).

MSCs are fibroblastic, rapidly proliferating, self-renewable, spindle-shaped, plastic adherent cells, which grow well in vitro (Gazdic et al. 2015). The absence of an MSC-specific marker enforced the International Society for Cellular Therapy (ISCT) to establish the basic criteria for phenotypic characterization of MSCs, which should be positive (≥95%) for at least three markers among CD29, CD44, CD73, CD90, and CD105 and negative for CD14 (expressed on monocytes), CD34 (expressed on hematopoietic cells), CD45 (pan-leukocyte marker), and CD79a and CD19 (markers of B lymphocytes) (Dominici et al. 2006). Importantly, MSCs do not express MHC class II proteins and costimulatory molecules, CD80 (B7–1), CD86 (B7–2), and CD40, suggesting their low immunogenicity and possibility for allogeneic transplantation in MHC-mismatched recipients (Gazdic et al. 2015; Volarevic et al. 2011).

One of the determining properties of MSCs is their capacity for spontaneous differentiation into osteocytes, chondrocytes, and adipocytes (Shariati et al. 2020). MSCs adopt different functional properties depending on their tissue source (Volarevic et al. 2011). Bone-marrow-derived MSCs have also been combined with neural stem cells to enhance their benefits for the treatment of spinal cord injury in an experimental animal model (Hosseini et al. 2018). AF-derived MSCs (AF-MSCs) exhibited a greater capacity for cell proliferation and self-renewal than BM-derived MSCs (BM-MSCs) (Harrell et al. 2018). AF-MSCs and PL-derived MSCs (PL-MSCs)) display *intracellular* and *extracellular markers* of pluripotent stem cells, such as octamer binding protein 3/4 (Oct-3/4), homeobox transcription factor Nanog, c-MYC, tumor-related antigen (TRA)-1–60, stage-specific embryonic antigen (SSEA)-3, and SSEA-4, and were capable of multilineage differentiation (Harrell et al. 2018). Several lines of evidence demonstrated that AF-MSCs and PL-MSCs might differentiate into functional neurons in vitro (Harrell et al. 2018). In comparison with BM-MSCs, AF-MSCs and PL-MSCs have a higher capacity for neural differentiation, more rapidly generate neurospheres, show higher expression of neural stemness markers (nestin, vimentin, Musashi), and produce a significantly higher amount of brain-derived growth factor (BDGF) and nerve growth factor (NGF), which enhance axonal regeneration (Harrell et al. 2018). Additionally, AF-MSCs and PL-MSCs may be a valuable cellular source of neural stem cells (NSCs) (Harrell et al. 2018).

NSCs derived from AF-MSCs showed better neuroregenerative properties than NSCs, which were differentiated from BM-MSCs (Harrell et al. 2018). Upon cultivation in a neuronal differentiation medium, AF-MSC-sourced NSCs generate more functional neurons than NSCs derived from BM-MSCs. Importantly, AF-MSCs and AF-MSC-derived NSCs maintained a normal karyotype in long-term cultures and were not tumorigenic in vivo after transplantation in immunodeficient mice and, accordingly, could be used in clinical settings (Harrell et al. 2018). AF-MSC-based biological product "derived Multiple Allogeneic Proteins Paracrine Signaling" (d-MAPPS) restored meibomian gland structure and function, improved tear stability, induced the regeneration of epithelial cells, and enhanced the repair of the ocular surface epithelial barrier in patients suffering from dry eye disease, meibomian gland dysfunction, and epithelial basement membrane dystrophy with recurrent corneal erosion syndrome (Harrell et al. 2018).

In addition to their potential for multilineage differentiation, MSCs are immunoregulatory cells that may inhibit activated immune cells, attenuate ongoing neuroinflammation, and create an immunosuppressive microenvironment, enabling the efficient repair and regeneration of injured and inflamed neural tissue (Shariati et al. 2020). MSCs, in a juxtacrine (cell-to-cell-contact-dependent manner) or paracrine manner (through the secretion of soluble and insoluble immunomodulatory factors), regulate the phenotype and function of all immune cells. This also includes the cells that play a crucially important pathogenic role in the development and progression of neurocognitive diseases (Harrell et al. 2021b; Andrzejewska et al. 2021; Shariati et al. 2020). MSC-sourced TGF-β, hepatic growth factor (HGF), and nitric oxide (NO) inhibit the proliferation and activation of T lymphocytes, NK cells,

and NKT cells. At the same time, MSC-derived indolamine 2,3-dioxygenase (IDO) promotes the generation and expansion of immunosuppressive CD4 + FoxP3 + Tregs by inducing the transdifferentiation of inflammatory Th17 cells in Tregs (Volarevic et al. 2017). By producing hemeoxygenase-1 (HO-1), prostaglandin E2 (PGE2), and anti-inflammatory cytokines (IL-10, IL-35, and IL-1 receptor antagonist (IL-1Ra)), MSCs generate a tolerogenic phenotype in DCs and induce the alternative activation of macrophages (Harrell et al. 2019a). Tolerogenic DCs inter-react with naïve T cells and promote their differentiation toward Tregs, while alternatively activated macrophages produce immunosuppressive cytokines, which create an anti-inflammatory environment and inhibit ongoing inflammation in neural tissue (Harrell et al. 2019b).

As part of their paracrine activity, MSCs produce various proangiogenic factors (basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), angiopoietin-1, placental growth factor (PGF), IL-6, monocyte chemotactic protein-1 (MCP-1), epidermal growth factor (EGF), HGF, and vascular endothelial growth factor (VEGF)), which induce neovascularization and provide trophic support to injured neurons (Volarevic et al. 2017; Haider and Aslam 2018).

Animal studies showed that MSCs may engraft in injured neural tissue and produce various bioactive factors suppressing the detrimental immune response and promoting neural regeneration and repopulation (Lv et al. 2021; Schweizer et al. 2020; Cooney et al. 2016). Importantly, the transplantation of allogeneic MSCs evoked little or no immune reactivity in MHC-mismatched recipient animals (Harrell et al. 2021a). Nevertheless, although MSCs, due to their regenerative, proangiogenic, and immunomodulatory properties, offer new hope in regenerative neurology, a variety of safety concerns have been raised regarding their clinical use (Volarevic et al. 2018).

Results from several clinical studies showed that MSC-based immunosuppressive effects were not noticed in all MSC-treated patients. Some MSC-treated patients did not respond to MSCs regardless of identical medical conditions to MSCs' responders. Even more, inflammation was aggravated in some of the MSC-treated patients (Volarevic et al. 2018). This phenomenon could be explained by the fact that MSCs are not strictly immunosuppressive cells and their immunoregulatory properties depend on the concentration of inflammatory cytokines (particularly TNF-α and IFN-γ) in the tissue in which they have been engrafted (Shariati et al. 2020). MSCs obtain an immunosuppressive or proinflammatory phenotype according to the inflammatory milieu to which they are exposed (Shariati et al. 2020). When MSCs are transplanted in the tissue with high levels of inflammatory cytokines, MSCs develop an immunosuppressive phenotype and induce the generation of tolerogenic phenotype in DCs, the alternative activation of macrophages, and the expansion of regulatory T lymphocytes, NK cells, and NKT cells (Shariati et al. 2020). When MSCs are engrafted in the microenvironment with low levels of TNF-α and IFN-γ, they obtain proinflammatory characteristics, produce large amounts of inflammatory mediators, and induce the activation of inflammatory immune cells (M1 macrophages, N1 neutrophils, Th1 and Th17 lymphocytes) (Shariati et al. 2020).

Additionally, MSCs' therapeutic potential is influenced by the donor's age (Haider 2018). When MSCs are considered for autologous clinical applications in the elderly, the possible age-related alterations in their phenotype and function, such as loss of proliferation and differentiation capacity and reduced therapeutic effectiveness, should be considered (Nurkovic et al. 2016).

Serious safety concerns are related to the possible unwanted differentiation of MSCs upon their engraftment in inflamed and injured tissues (Musiał-Wysocka et al. 2019). Encapsulated formations, such as calcifications and ossifications, were detected in the tissue in which MSCs were transplanted, suggesting an unintended osteogenic and chondrogenic differentiation of engrafted MSCs under the influence of the local microenvironment (Volarevic et al. 2018). Furthermore, when MSCs had been given, together with immunosuppressive medications (which have been used as standard therapy for autoimmune and neuroinflammatory diseases), several patients developed severe, life-threatening respiratory and gastrointestinal infections. These observations provide evidence that MSCs could not be used along with other immunosuppressive drugs. Additionally, many chromosomal abnormalities were observed in some cultures of long-lived MSCs, indicating that a detailed genetic analysis of MSCs has to be done before their transplantation into patients (Volarevic et al. 2018).

Since numerous experimental and clinical trials showed that the majority of the immunoregulatory and angiomodulatory effects of MSCs in the treatment of neurocognitive disorders were dependent on the activity of MSC-sourced bioactive factors (lipids, proteins (enzymes, cytokines, chemokines, immunoregulatory proteins, trophic and growth factors, and microRNAs (miRNAs)), the therapeutic use of these soluble mediators (either alone or within exosomes (Exos)) hold a great promise in cell-free therapy of neurocognitive diseases (Harrell et al. 2019a; Haider and Aramini 2020). MSC-derived exosomes (MSC-Exos) contain all of MSC-sourced neuroprotective and immunomodulatory factors and, thanks to their nano-sized dimension and lipid envelope, may easily penetrate the blood-brain barrier and brain tissue and reach target cells (Harrell et al. 2021a). As cell-free therapeutic agents, MSC-Exos avoid all safety concerns related to the transplantation of MSCs, including unwanted differentiation, undesired immunoregulation, and malignant transformation (Harrell et al. 2021a; Volarevic et al. 2018). Accordingly, a large number of recently published experimental studies showed advantageous benefits of MSC-Exos over MSCs in the treatment of AD, PD, posttraumatic and ischemic brain injury, schizophrenia, and autism spectrum disorders (ASDs) (Harrell et al. 2021).

Therapeutic Effects of MSCs and Their Exosomes in the Treatment of Alzheimer's Disease

Alzheimer's disease (AD) is a neurological disorder that provokes memory loss, cognitive dysfunction, abnormal behavior, and reduced daily functions of patients suffering from it (Nurkovic et al. 2016). Patients with advanced AD often enter a

coma and pass away due to exhaustion and incapacitation (Joe and Ringman 2019). The main neuropathological characteristics of AD are the formation of neurofibrillary tangles (hyperphosphorylated microtubule-associated protein tau-intraneuronal aggregates) and the deposition of amyloid plaques (insoluble deposits of amyloid peptide) (Jellinger 2020). They are commonly detected in the medial temporal lobe and hippocampus of the brain. The intraneuronal aggregation of hyperphosphorylated tau proteins leads to microtubule disintegration, causing cell death in affected neurons, while amyloid peptides accumulate and inhibit synaptic signal transmission (Jellinger 2020).

Results obtained in a large number of preclinical studies showed that MSCs efficiently alleviate AD-related symptoms in experimental animals by decreasing the deposition of amyloid β protein and increasing the levels of acetylcholine in the brain, which led to the better survival of neuronal cells and, consequently, resulted in an improved spatial learning memory of AD animals (Harrell et al. 2021b; Andrzejewska et al. 2021; Si and Wang 2021; Shariati et al. 2020; Qin et al. 2020). MSCs reduce the deposition of amyloid β protein by inducing the autophagy of pathological neurons, which had increased the accumulation of this protein (Harrell et al. 2021b). Additionally, MSCs improve the neurogenesis and synaptogenesis of hippocampal neurons in AD animals by producing a large number of neurotrophins and growth factors (BDGF, nerve growth factor (NGF), VEGF) (Andrzejewska et al. 2021). MSCs reduced synaptic protein loss by restoring the levels of synaptotagmin-1, synaptophysin, and glutamic acid decarboxylase-65 in the hippocampus of AD rats. Additionally, by modulating the expression and activity of choline acetyltransferase and acetylcholinesterase, MSCs increase levels of acetylcholine in the hippocampus, which is followed by a significant improvement in the learning abilities and cognitive function of AD animals (Andrzejewska et al. 2021).

In addition to their direct effect on pathological neurons, MSCs prevent AD progression by suppressing ongoing inflammation by inhibiting inflammatory M1 microglia cells (Andrzejewska et al. 2021). MSCs inhibit the secretion of inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, macrophage chemotactic protein (MCP)) and induce the production of immunosuppressive cytokines (TGF-β, IL-10) in microglia cells, favoring their transdifferentiation from inflammatory (M1) into anti-inflammatory (M2) cells (Andrzejewska et al. 2021). Accordingly, a cross-talk between MSCs and microglia cells resulted in the alleviation of inflammatory and the upregulation of immunosuppressive cytokines in the brains of AD animals, which was manifested by the significant improvement in cognitive functions (Bagheri-Mohammadi 2021).

Results obtained in various experimental trials demonstrated that exosomes are responsible for the interneuronal transfer of hazardous chemicals within the brain of AD patients, playing an essential role in the progression and aggravation of AD (Beatriz et al. 2021). In line with these findings, several research groups investigated whether MSC-Exos may use the same pathways (gap junctions, synaptic transmission, endosomal/lysosomal secretion system) to deliver MSC-derived neuroprotective and trophic chemicals to injured neurons, resulting in the alleviation of AD-related symptoms (Harrell et al. 2021b; Nakano and Fujimiya 2021;

Guo et al. 2020; Yang et al. 2020b; Yin et al. 2020; Chakari-Khiavi et al. 2019). As expected, MSC-Exos are effective in treating AD in a large number of animal studies (Harrell et al. 2021; Nakano and Fujimiya 2021; Guo et al. 2020, Yang et al. 2020b; Yin et al. 2020; Chakari-Khiavi et al. 2019).

Wang and Yang demonstrated that intravenously injected BM-MSC-Exos significantly reduced inflammation and the accumulation of amyloid peptides and restored synaptic signal transmission in the brains of APP/PS1 transgenic mice, a well-established animal model of AD (Wang and Yang 2020). The administration of BM-MSC-Exos significantly improved cognitive function in experimental mice by stimulating the sphingosine kinase (S1K)/sphingosine-1-phosphate (S1P) signaling pathway in the central nervous system (Wang and Yang 2020). S1K and S1P regulate sphingomyelin metabolism, maintain vascular solidity, and encourage the development of blood vessels in the brain, enabling a more efficient transfer of neurotrophins to the injured neurons (Jellinger 2020). Since a downregulated expression of S1K and/or S1P was noticed in the brain tissue samples of APP/S1 mice and AD patients (Jellinger 2020), BM-MSC-Exo-induced restoration of S1K/S1P signaling led to the significant improvement of cognitive function and the alleviation of AD-related symptoms in APP/S1 animals (Wang and Yang 2020). Increased S1K and S1P expression was fully prevented by the intraperitoneal injection of S1K or S1P inhibitors, implying that the therapeutic effects of BM-MSC-Exos in AD were dependent on the activation of S1/S1P signaling pathways in the brain of APP/S1 mice (Wang and Yang 2020).

In addition to the modulation of S1K/S1P signaling, MSC-Exo-based neuroprotection in APP/PS1 mice was also based on MSC-Exo-dependent activation of the amyloid-peptide-degrading enzyme neprilysin (Ding et al. 2018; Elia et al. 2019). Intracerebral injection of BM-MSC-Exos increased neprilysin expression and activity, which reduced amyloid protein accumulation in APP/PS1 animals (Ding et al. 2018). BM-MSC-Exos also reduced the activity of the β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and downregulated the expression of presenilin-1 (PS1), both of which are required for the synthesis and degradation of amyloid precursor proteins (Harrell et al. 2021b; Elia et al. 2019). Additionally, BM-MSC-Exo-based therapy significantly increased the expression of NeuN (a neuronal cell biomarker). It also led to the increased accumulation of NeuN in the cortex and hippocampus of APP/S1 mice (Harrell et al. 2021b; Elia et al. 2019). As a result of MSC-Exo-based therapy, the total number of dystrophic neurites in the cortex and hippocampus of BM-MSC-Exo-treated APP/PS1 mice was considerably lower than in untreated animals (Elia et al. 2019).

Notably, according to Reza-Zaldivar and colleagues, MSC-Exos restored cognitive performance in AD mice in the same way that parental MSCs did (Reza-Zaldivar et al. 2019). The Morris water maze (MWM) and novel object recognition (NOR) tests revealed that injecting either MSC-Exos or MSCs into AD rats effectively reduced cognitive impairment (Reza-Zaldivar et al. 2019). There was no difference in the benefits achieved by MSCs and MSC-Exos in terms of learning ability and memory loss (Harrell et al. 2021b; Andrzejewska et al. 2021; Reza-Zaldivar et al. 2019). The MWM test revealed that both MSC-Exos and MSCs

dramatically improved learning ability in AD animals. The NOR test revealed that MSC-Exo-treated and MSC-treated mice had equivalent increases in the percentage of interaction time when compared to untreated mice, implying that MSCs restored learning ability and memory in AD mice in a paracrine manner by delivering immunomodulatory and trophic factors into the injured neurons of the affected brain via exosomes (Reza-Zaldivar et al. 2019). Accordingly, Reza-Zaldivar and colleagues concluded that MSC-Exos were mainly responsible for the beneficial effects of MSCs in AD, particularly for MSC-dependent improvement of cognitive function (Reza-Zaldivar et al. 2019).

Similarly, as observed after the injection of BM-MSC-Exos, AT-derived MSC-Exos (AT-MSC-Exos) could efficiently provide neuroprotection, stimulate neurogenesis, and alleviate cognitive impairment in APP/PS1 mice (Ma et al. 2020). AT-MSC-Exos reach the brain quickly after intranasal delivery, accumulating in neurons and glial cells. AT-MSC-Exo-treated APP/PS1 mice showed significantly reduced amyloid β protein accumulation and decreased microglia activation (Ma et al. 2020). Importantly, the proteomic analysis of AT-MSC-Exos revealed the presence of neurotrophins (filamin A, vinculin, neuropilin-1, neuroplastin, glia-derived nexin, flotillin-1, drebrin, teneurin-4), which induced neurogenesis and myelin formation, stimulated axonal growth and regeneration, and provided neuroprotection to injured neurons. In AT-MSC-Exo-treated neurons, 1094 genes were upregulated, while 267 genes were downregulated (Ma et al. 2020). Raised expression of PCLO, TENM1, and NEXMIF genes, which regulated synaptic function and improved memory in experimental mice and reduced the expression of the BAD gene, which caused the cell death of injured neurons, were found in AT-MSC-Exos. As a result, the injection of AT-MSC-Exos reduced neurologic damage, increased the total number of newly produced neurons, and effectively corrected memory deficits in APP/PS1 mice (Ma et al. 2020).

The accumulation of amyloid protein is aggravated by microglia-driven neuroinflammation, which plays a key role in the genesis and progression of AD (Subramaniyan and Terrando 2019). Several research groups demonstrated that the main mechanism for MSC-Exo-based reduction of cognitive impairment in APP/PS1 mice is the suppression of microglia's proinflammatory characteristics (Harrell et al. 2021; Andrzejewska et al. 2021; Bagheri-Mohammadi 2021; Ding et al. 2018). By using APP/PS1 mice as a model for AD, Ding and colleagues showed that the administration of umbilical-cord-derived MSC-Exos (UC-MSC-Exos) inhibited the generation of inflammatory phenotypes in activated microglia, which, consequently, resulted in alleviated inflammation in the brain and was manifested by improved learning ability and memory of experimental animals (Ding et al. 2018). UC-MSC-Exo-treated APP/PS1 mice had a significantly shorter mean escape latency, a greater number of platform location crossing times, and a longer time spent in the target quadrant than MSC-Exo-untreated mice, implying that UC-MSC-Exos improved APP/PS1 mice's behavioral performance by improving spatial learning and memory function (Ding et al. 2018). Additionally, UC-MSC-Exos considerably increased the activity of amyloid-β-degrading enzymes (neprilysin- and insulin-degrading enzymes) in the brains of experimental mice, resulting in a considerable

reduction in amyloid β deposition (Ding et al. 2018). The immunostaining of the brains of APP/S1 mice revealed a significantly reduced number of Iba-1-positive inflammatory microglia cells in the brains of UC-MSC-Exo-treated mice (Ding et al. 2018). The cellular makeup of microglial cells revealed that most microglia cells were polarized toward the immunosuppressive M2 phenotype (Ding et al. 2018). Significantly increased number of chitinase 3-like 3 (YM-1), arginase-1 (Arg-1), mannose receptors C type 1 (MRC1), and haptoglobin/hemoglobin scavenger receptor (CD163)-expressing M2 microglia cells were noticed in the brains of UC-MSC-Exo-treated APP/S1 mice compared to the UC-MSC-Exo-untreated animals (Harrell et al. 2021b; Ding et al. 2018). As a result, levels of M2 microglia-derived immunosuppressive cytokines (TGF-β and IL-10) increased in the peripheral blood and brains of UC-MSC-Exo-treated APP/PS1 mice, while levels of M1 microglia-sourced pro-inflammatory cytokines (TNF-α and IL-1β) decreased, confirming that the significant improvement of cognitive function in UC-MSC-Exo-treated APP/S1 mice was mainly a consequence of UC-MSC-Exo-based alleviation of microglia-driven neuroinflammation (Harrell et al. 2021; Ding et al. 2018).

Results obtained by Nakano and colleagues, who emphasized the crucial role of MSC-sourced miRNA-146 for MSC-Exo-dependent reduction of microglia and neuroinflammation in AD mice (Nakano et al. 2020), were consistent with these findings. MiRNA-146 is a noncoding RNA molecule that regulates microglia inflammatory characteristics (Nakano and Fujimiya 2021). In microglia, MSC-Exos suppressed TNF-receptor-associated factor 6 (TRAF6) and IL-1-receptor-associated kinase 1 (IRAK1) in a miRNA-146-dependent way, resulting in decreased phosphorylation of transcriptional factor NF-kB (Nakano and Fujimiya 2021).

In MSC-Exo-treated microglia, the inhibition of the NF-kB signaling pathway reduced the production of inducible nitric oxide synthase (iNOS), TNF-α, IL-1β, and IL-6 genes. It prevented the inflammatory M1 phenotype (Nakano et al. 2020). M1 microglia release NO and proinflammatory cytokines (TNF-α, IL-1β) that alter synaptogenesis and decrease cognitive function, leading to the progression of AD (Subramaniyan and Terrando 2019). Intracerebroventricularly injected BM-MSC-Exos alleviated neuroinflammation and improved spatial learning and memory function in APP/PS1 mice by suppressing M1 microglia activation and promoting their differentiation into M2 immunosuppressive cells in a miRNA-146-dependent manner (Nakano et al. 2020; Wang et al. 2018).

MSC-derived miRNA-21, in addition to miRNA-146, was also responsible for MSC-Exo-based immunomodulation in the inflamed brains of APP/PS1 mice (Cui et al. 2018). Exosomes derived from hypoxia-preconditioned MSCs, in a miRNA-21-dependent way, suppressed microglial cell activation in APP/PS1 mice and prevented memory impairments (Cui et al. 2018). Significantly increased miRNA-21 corresponded to reduced amyloid β protein deposition, downregulated concentrations of inflammatory cytokines, reduced activation of transcriptional factors (signal transducer and activator of transcription 3 (STAT3) and NF-kB), and increased levels of anti-inflammatory cytokine IL-10 in the brains of MSC-Exo-treated APP/PS1 mice (Cui et al. 2018).

Even though MSC-Exos can cross the blood-brain barrier, most intravenously injected MSC-Exos end up in the spleen and liver (Offen et al. 2019; Morishita et al. 2017). Cui and colleagues used MSC-Exos conjugated with the neurotropic rabies virus glycoprotein (RVG; RVG-tagged MSC-Exos) to target intravenously injected MSC-Exos into the brains of APP/PS1 mice (Cui et al. 2019). RVG-tagged MSC-Exos showed enhanced tropism for the cortex and hippocampus compared to RVG-untagged MSC-Exos. Accordingly, a higher presence of MSC-Exos was observed in the brains of APP/PS1 animals that received RVG-tagged MSC-Exos (Cui et al. 2019). The injection of RVG-tagged MSC-Exo dramatically reduced the serum levels of inflammatory cytokines (TNF-α, IL-β, and IL-6) and increased the serum concentration of immunosuppressive IL-10 in APP/PS1 mice. As a result, the behavioral and cognitive skills of APP/PS1 mice that received RVG-tagged MSC-Exos were much better than the learning and memory abilities of animals that were treated with RVG-untagged MSC-Exos (Cui et al. 2019).

The encouraging results of experimental studies raised hope for an efficient clinical application of MSCs in AD therapy (Andrzejewska et al. 2021). Initially, in 2011, scientists from South Korea conducted a Phase I clinical trial in which nine patients with mild to moderate AD underwent stereotactic brain infusion of allogeneic MSCs (ClinicalTrials identifier: NCT01547689). The transplanted MSCs were well tolerated without any significant side effects related to their administration. Subsequently, Phase I/II clinical trials in which AD patients received allogeneic MSCs in the hippocampal region were also reported to be safe since no patients reported serious complications during 18–24 months of follow-up (ClinicalTrials identifier: NCT01696591 and ClinicalTrials identifier: NCT02054208) (Andrzejewska et al. 2021). The first Phase II clinical trial that investigated the therapeutic potential of MSCs in the treatment of 40 AD patients was conducted in the United States in 2015 (ClinicalTrials identifier: NCT02833792) (Andrzejewska et al. 2021). Similar trials have been elicited in Europe and Asia, but results proving the therapeutic efficacy of MSCs in the alleviation of AD-related signs and symptoms are still waiting to be published.

Improvement of Cognitive Impairment Following Brain Damage and Ischemia in MSC-Treated Mice

The hippocampus is required for memory, spatial learning, and cognition (Shetty 2014). Accordingly, due to hippocampus injury, cognitive impairment and memory loss emerge (Shetty 2014). Traumatic injury and ischemia cause temporary neurogenesis, a compensatory response that should help injured neurons recover their function. However, the injured brain's regenerating ability is severely restricted (Chen et al. 2020b).

Intrathecal, intravenous, and intra-arterial infusion of MSCs significantly improved the cognitive deficit in experimental animals suffering from ischemia or traumatic brain injury (TBI) (Andrzejewska et al. 2021). MSCs, delivered directly into the injured or ischemic brains, engrafted in the inflamed microenvironment,

obtained an anti-inflammatory phenotype and produced large amounts of neurotrophins (neuropilin-1, neuroplastin, glia-derived nexin, flotillin-1), immunosuppressive cytokines (IL-10, IL-35, TGF-β), and proangiogenic factors (VEGF, hypoxia-inducible factor 1α (HIF-1 α)), which attenuated ongoing inflammation, promoted neovascularization, and finally led to the regeneration of injured neurons (Andrzejewska et al. 2021). Systemically injected MSCs usually engraft in lungs and liver (Volarevic et al. 2011). By releasing various neurotrophins, MSCs, in endocrine manner, modulate phenotype and function of injured neurons and microglial cells (Volarevic et al. 2011). Moreover, MSCs may, in a juxtacrine and paracrine manner, stimulate the microglial cells to secrete trophic factors, which will further promote neurogenesis, neuroprotection, and neural repair in ischemic and injured brains (Bagheri-Mohammadi 2021). MSCs generate the M2 phenotype in microglia, characterized by an increased capacity for enhanced production of neurotrophins and immunosuppressive cytokines (Andrzejewska et al. 2021; Bagheri-Mohammadi 2021). As a result, transplanted MSCs and M2 microglia create an immunosuppressive microenvironment with an increased concentration of neuroprotective molecules and growth factors that prevent the apoptosis of injured neurons and stimulate their regeneration (Andrzejewska et al. 2021; Bagheri-Mohammadi 2021).

The genetic modification of MSCs improves their therapeutic effects in neuroprotection and immunomodulation after transplantation in ischemic and injured brains (Andrzejewska et al. 2021). Genetically engineered MSCs that overexpress fibroblast growth factor 21 (FGF-21) or chemokine receptor CXCR4 enhanced cell homing to the site of brain injury and inflammation (Andrzejewska et al. 2021). A significant increase in engrafted MSCs resulted in increased neurogenesis in injured brains, particularly in the hippocampal region. As a result, significantly improved cognitive function and learning abilities were observed in TBI rats that received FGF-21 or CXCR4-overexpressing MSCs (Andrzejewska et al. 2021). Similarly, genetically engineered MSCs that overexpress IL-4 and IL-10 were superior in the inhibition of Th1 and Th17 cell-driven neuroinflammation, while HIF-1 α-overexpressing MSCs remarkably increased neo-angiogenesis in ischemic brains, contributing to the better restoration of cognitive function in MSC-treated TBI rats (Andrzejewska et al. 2021).

By using the same molecular mechanisms as their parental MSCs, MSC-Exos block harmful immune responses and enhance neurogenesis and neuritogenesis by delivering trophic, vasoactive, and immunomodulatory molecules to damaged neurons and microglia (Harrell et al. 2021a; Bagheri-Mohammadi 2021; Chen et al. 2020). Several lines of evidence showed that MSC-Exos effectively restored cognition, learning disabilities, and memory loss in injured and ischemic hippocampal neurons (Harrell et al. 2021b; Chen et al. 2020c; Niu et al. 2020; Kim et al. 2016; Zhang et al. 2015, 2020; Gao et al. 2020; Yang et al. 2020a).

By using an experimental murine model of acute brain injury, Niu and colleagues demonstrated that the intravenous injection of UC-MSC-Exos improved cognitive performance in experimental animals by modulating metabolism in hippocampal neurons (Niu et al. 2020). Proteomic analysis showed that UC-MSC-Exos contain 67 proteins that are able to alter metabolic function in damaged neurons

(Niu et al. 2020). Among these MSC-derived proteins, adiponectin was considered the most important regulator of metabolism. Accordingly, adiponectin levels in the hippocampus and serum samples of UC-MSC-Exo-treated mice were found to be higher, and an increase in adiponectin levels was linked to the better cognitive function of UC-MSC-Exo-treated animals (Niu et al. 2020).

Kim and coworkers used an experimental murine model of TBI to demonstrate the therapeutic potential of MSC-Exos in the restoration of cognitive function after TBI (Kim et al. 2016). They showed that only a few days after their intravenous injection, MSC-Exos significantly corrected pattern separation and spatial learning defects in experimental animals (Kim et al. 2016).

A single intravenous injection of MSC-Exos dramatically restored sensorimotor and cognitive performance in rats with unilateral mild cortical contusion (Zhang et al. 2020). There was a remarkable reduction in hippocampus neuronal cell death, alleviated neuroinflammation, and a significant rise in the number of newly produced blood vessels and neurons in the brains of MSC-Exo-treated rats. MSC-Exos improved animals' cognitive function by regulating microglia activation and preventing reactive astrogliosis, which led to a significant reduction in the inflammation-induced injury of neurons (Zhang et al. 2020). Additionally, MSC-Exos were able to correct myelination deficiencies and abnormalities of white matter. All of these MSC-Exo-based improvements in neural cells' morphology and function were followed by restored cognition and learning abilities of experimental animals (Zhang et al. 2020).

Similar to these findings are the results reported by Zhang and colleagues, who found that a systemic injection of MSC-Exos managed to significantly improve cognitive function in rats with TBI by encouraging functional recovery and neurovascular remodeling (Zhang et al. 2015). The MWM test showed that MSC-Exos-treated TBI rats had considerable improvement in spatial learning when compared to saline-treated animals (Zhang et al. 2015). Additionally, a significant increase in the number of newly formed immature and mature neurons in the dentate gyrus of MSC-Exo-treated TBI rats was accompanied by an alleviated concentration of inflammatory cytokines and an increased number of newly generated blood vessels in the brains, indicating that MSC-Exo-induced improvement in the cognitive function of TBI rats was due to the activity of MSC-Exo-sourced immunosuppressive, neurotrophic, and proangiogenic factors (Zhang et al. 2015).

Gao and colleagues suggested that miRNA-21, already reported for its anti-apoptotic activity (Haider et al. 2010), was mostly responsible for MSC-Exos' positive effects in the restoration of cognitive function following brain injury. In experimental rats, MSC-Exo-delivered miRNA-21 protected neurons from apoptosis and reduced subarachnoid hemorrhage (SAH)-induced cognitive impairment (Gao et al. 2020). MSC-Exo-delivered miRNA-21 inhibited apoptosis in damaged neurons by activating the PTEN/Akt signaling pathway (Gao et al. 2020). The knockdown of miRNA-21 as well as the administration of a PTEN/Akt inhibitor completely diminished MSC-Exo-dependent neuroprotection, demonstrating a critical role of miRNA-21/PTEN/Akt signaling for MSC-Exo-based restoration of cognitive function following SAH (Gao et al. 2020).

By delivering proangiogenic and trophic factors to the ischemic regions of injured brains, MSC-Exos prevented ischemia-induced apoptosis of hippocampal neurons and significantly reduced the cognitive impairment of experimental animals (Yang et al. 2020). MSC-Exos reduced the overall number of dead neurons and increased neuronal density in the ischemia boundary zone by downregulating the expression of proapoptotic proteins Bax, caspase-3, and caspase-9 and by increasing the expression of antiapoptotic protein Bcl-2 (Yang et al. 2020a).

Hippocampal brain injury and cognitive impairment are caused by ischemic injury, oxidative stress, glucose metabolism abnormalities (hyper- or hypoglycemia), alterations in glutamate neurotransmission, and impaired hippocampal synaptic plasticity in diabetic individuals (Hamed 2017).

Since MSC-Exos contain a large number of neurotrophins, proangiogenic factors, and molecules that control metabolism in ischemic neurons, several research groups analyzed the therapeutic potential of MSC-Exos in the treatment of diabetes-induced cognitive dysfunction (Nakano et al. 2016; Zhao et al. 2019; Kubota et al. 2018). Zhao and colleagues discovered that diabetic mice treated with BM-MSC had a practically complete recovery of cognition. In the hippocampus of diabetic mice, the intracerebroventricular injection of BM-MSC-Exos dramatically reduced the degeneration and synaptic loss of neurons (Zhao et al. 2019). MSC-Exos decreased inflammation-induced damage of hippocampal neurons by downregulating the expression of IRAK1 kinase in a miRNA-146-dependent manner (Zhao et al. 2019). By delivering immunoregulatory miRNA-146 in astrocytes, MSC-Exos suppressed the activation of IRAK1 kinase and altered the inflammatory phenotype and function of astrocytes. The inhibition of IRAK1 prevented the activation of transcriptional factor NF-kB, which resulted in the alleviated production of proinflammatory cytokines (TNF-α and IL-1β) in astrocytes and, consequently, led to the alleviation of ongoing inflammation (Zhao et al. 2019). Attenuated inflammation resulted in a decreased loss of hippocampal neurons, manifested by the significantly improved cognitive function and learning abilities of MSC-Exo-treated animals (Zhao et al. 2019).

Therapeutic Effects of MSCs and MSC-Exos in the Treatment of Parkinson's Disease, Schizophrenia, and Autism

Parkinson's disease (PD) is a progressive neurocognitive disease characterized by a loss of dopaminergic neurons (McDermott et al. 2018; Kubota et al. 2018). MSCs are effective in the treatment of PD in various experimental studies (Hwang et al. 2018; Chen et al. 2020a). The intracerebral injection of MSCs significantly improved locomotor activity and enhanced neurogenesis in PD animals (Andrzejewska et al. 2021). A remarkably increased number of tyrosine hydroxylase (TH)-positive dopaminergic neurons and elevated dopamine levels in the striatum were observed in MSC-treated PD animals. MSC transplantation promoted the survival of dopaminergic neurons by inducing the enhanced expression of antiapoptotic Bcl-2 protein and suppressing the activity of proapoptotic Bax protein in these cells (Andrzejewska et al. 2021; Chen et al. 2020a). Additionally, MSCs

delivered several neurotrophic factors (brain-derived neurotrophic factor (BDNF), cerebral dopamine neurotrophic factor (CDNF), HGF, and neurotrophin-3) to the dopaminergic neurons, enhancing their viability and regeneration (Chen et al. 2020b). Finally, an increased number of immunosuppressive M2 and the reduced presence of inflammatory M1 microglial cells were observed in the brains of MSC-treated PD mice, indicating that the beneficial effects of MSCs in the alleviation of PD-related symptoms were, at least partially, relied on in the MSC-dependent suppression of microglia-driven inflammation (Andrzejewska et al. 2021; Bagheri-Mohammadi 2021; Chen et al. 2020c).

Since a majority of MSC-based beneficial effects of PD treatment relied on the activity of MSC-sourced bioactive molecules (Harrell et al. 2021b; Andrzejewska et al. 2021), Chen and colleagues investigated whether intravenously given MSC-Exos may cause the regeneration of dopaminergic neurons in the same manner as their parental cells (Chen et al. 2020b). Systemically infused MSC-Exos easily crossed the blood-brain barrier and reached dopaminergic neurons in the substantia nigra of PD rats (Chen et al. 2020b). The histological examination of brain tissue samples revealed that MSC-Exos prevented the apoptosis of dopaminergic neurons (Chen et al. 2020b). Importantly, MSC-Exos dramatically increased levels of dopamine and its metabolites (dihydroxyphenylacetic acid and homovanillic acid) in the striatum, implying that MSC-Exo-based therapy managed to significantly improve dopaminergic neuron activity in PD mice.

Autism spectrum disorders (ASDs) represent a group of neurodevelopmental diseases characterized by cognitive impairment, increased repetitive behaviors, and difficulties in communication and social interaction (Hodges et al. 2020). The therapeutic potential of MSC-Exos in the therapy of ASD has been explored in BTBR T + tf/J mice, which have a behavioral phenotype comparable to human ASD (reduced social approach, low reciprocal social interactions, and impaired juvenile play) (Perets et al. 2018). Perets and colleagues demonstrated that the intranasal injection of MSC-Exos considerably reduced autism-like behavior and ASD-related symptoms in BTBR T + tf/J mice (Perets et al. 2018). Significantly enhanced male-to-male social interaction and reduced repetitive behaviors during social interaction were found in MSC-Exo-treated BTBR T + tf/J mice (61). Additionally, MSC-Exo-treated BTBR T + tf/J mice had more sophisticated and longer male-to-female ultrasonic vocalization, making them more similar to the healthy mice of the control group (Perets et al. 2018).

The pup retrieval behavior of female BTBR T + tf/J mice was also greatly improved by MSC-Exos. While saline-treated BTBR T + tf/J females returned just 2 of 24 pups to the nest, MSC-Exo-treated BTBR T + tf/J females returned all (18/18) pups, suggesting a considerable increase in maternal behavior (Perets et al. 2018).

The intranasal injection of MSC-Exos significantly improved the cognitive function of phencyclidine (PCP)-treated mice, used as a well-established murine model of schizophrenia. Tsivion-Visbord and colleagues found that MSC-Exos reduced schizophrenia-like behaviors by improving the survival of gamma-aminobutyric acid (GABA)-producing neurons (Tsivion-Visbord et al. 2020). Immediately after intranasal injection, the majority of MSC-Exos were observed in the neurons of the

prefrontal cortex (PFC), the brain area most severely impaired in schizophrenia. A significantly higher presence of GABA-producing PFC neurons was accompanied by decreased levels of glutamate in the cerebrospinal fluid of MSC-Exo + PCP-treated animals. MSC-Exo-based reduction of schizophrenia-like behavior was evidenced by improved social interaction and disrupted prepulse inhibition in PCP-treated mice (Tsivion-Visbord et al. 2020). Since the intranasal delivery of MSC-Exos was well tolerated and no side effects were observed in MSC-Exo-treated mice (Perets et al. 2018; Tsivion-Visbord et al. 2020), the efficacy of this noninvasive therapeutic approach for the improvement of cognitive function should be further investigated during upcoming clinical trials.

MSC-Exos play a crucial role in the information transfer between MSCs and recipient cells (neurons and microglia) (Harrell et al. 2021b). MSC-sourced miRNAs, trophic factors, enzymes, and immunomodulatory and proangiogenic chemicals delivered by MSC-Exos into the neurons and microglia enhance neurogenesis and reduce inflammation-induced damage of hippocampal neurons, improving the cognitive function of MSC-Exo-treated animals (Harrell et al. 2021b). Importantly, the capacity for immunomodulation and neuroprotection of MSC-Exos was comparable to or even superior to that mediated by their parental MSCs (Harrell et al. 2021b; Andrzejewska et al. 2021). The effects of MSC-Exos were unaffected by the local tissue's microenvironment. Unlike MSCs, which may change their phenotype and function as a result of engraftment in diverse tissue microenvironments, MSC-Exos deliver their cargo (immunoregulatory molecules and growth factors) directly to the target cells, and their therapeutic potential is not affected by local tissue milieu (Harrell et al. 2021a; Andrzejewska et al. 2021). Moreover, the composition of exosomal cargo can be modified by the physical or genetic modification of MSCs to achieve the desired prognosis (Haider and Aramini 2020).

Nevertheless, despite these promising outcomes, it has to be noted that various concerns must be addressed before MSC-Exos can be widely used in clinical settings on a regular basis (Harrell et al. 2021a). Since a variety of antiapoptotic and immunosuppressive molecules have been hypothesized as critical for the MSC-Exo-based restoration of cognitive function, more experimental research is needed to prove those findings and to delineate which of these factors play a critical role in MSC-Exo-based immunomodulation and neuroregeneration. Moreover, before MSC-Exos could be offered as a universal human therapeutic agent, future experimental studies and clinical trials should identify the exact disease-specific therapeutic dose, route of administration, and schedule for MSC-Exos-based therapy (Harrell et al. 2021).

Conclusion

MSCs produce a large number of immunoregulatory, proangiogenic, and trophic factors, which may suppress detrimental immune responses, alleviate ongoing neuroinflammation, improve oxygen supply, and induce the regeneration of injured neurons (Harrell et al. 2021a; Andrzejewska et al. 2021). The transplantation of MSCs as well as the infusion of MSC-Exos enhance hippocampal neurogenesis,

stabilize synapses, and induce the generation of anti-inflammatory and immunosuppressive phenotypes in microglia cells within injured and inflamed brains. Accordingly, a significantly reduced loss of hippocampal neurons and a remarkable improvement in cognitive function was noticed in MSCs and MSC-Exo-treated animal models of AD, TBI, PD, ASDs, and schizophrenia (Harrell et al. 2021b; Andrzejewska et al. 2021). The encouraging results of experimental studies raised hope for an efficient clinical application of MSCs. The results obtained in already conducted clinical trials demonstrated that MSC-based therapy is a safe therapeutic approach that does not cause undesirable severe side effects (Andrzejewska et al. 2021). However, the therapeutic efficacy of MSCs or MSC-Exos in the treatment of neurocognitive diseases has not yet been confirmed in clinical settings, and results of upcoming clinical trials that analyze their therapeutic potential are expected with enthusiasm and sincere hope.

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Augmenting Mesenchymal Stem Cell-Based Therapy of the Infarcted Myocardium with Statins

15

Sulaiman Alnasser, Mabrouk AL-Rasheedi, Mateq A. Alreshidi, Saleh F. Alqifari, and Khawaja Husnain Haider

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S. Alnasser
Pharmacology and Toxicology Department, Unizah College of Pharmacy, Qassim University, Unaizah, Kingdom of Saudi Arabia
e-mail: sm.alnasser@qu.edu.sa

M. AL-Rasheedi
Al Bukayriah General Hospital, AlQassim, Kingdom of Saudi Arabia

M. A. Alreshidi
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukayriyah, Kingdom of Saudi Arabia
e-mail: ma.alreshidi@sr.edu.sa

S. F. Alqifari
College of Pharmacy - Department of Pharmacy Practice, University of Tabuk, Tabuk, Saudi Arabia
e-mail: s.alqifari@sr.edu.sa

K. H. Haider (✉)
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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Abstract

Preconditioning (PC) affords the most potent cytoprotective effects ever known in the physiological system. Since the publication of the first report that preconditioning by cyclical exposure to intermittent sublethal ischemia-reperfusion episodes is cardioprotective, the strategy has been extrapolated to sustain its beneficial effects at cellular and subcellular levels. The underlying principle of preconditioning is that exposure to sublethal episodes of a noxious stimulus triggers survival signaling pathways that render the cells resistant to subsequent exposure to the lethal stimulus. Diverging from the classical protocol involving treatment with ischemia-reperfusion, various preconditioning strategies have been developed and optimized to include physical, chemical, genetic, and pharmacological manipulation of cells to mimic the effects of ischemic preconditioning. Besides survival signaling, such manipulations significantly impact the stemness characteristics, paracrine behavior, angiogenic and differentiation potential, and various other aspects of cellular biology. Primarily used as cholesterol-lowering drugs, statins constitute a group of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors that have been effectively employed as preconditioning mimetics. Various members of the statin group, natural, semisynthetic, or synthetic, i.e., lovastatin, simvastatin, atorvastatin, rosuvastatin, etc., have been used to exploit their pleiotropic effects on the stem/progenitor cells as a part of the preconditioning strategy to enhance their stemness and functionality postengraftment. This book chapter provides a critical review of the advancements in pharmacological preconditioning of stem/progenitor cells in general and with the use of statin in particular. It highlights the mechanism that renders superiority in the use of statins as preconditioning mimetics.

Keywords

Angiogenesis · Heart · Pharmacological · Preconditioning · Statins · Stem cells · Survival

List of Abbreviations

AMI	Acute myocardial infarction
AMPK	Adenosine monophosphate-activated protein kinase
Bax	Blc2-associated X-protein
Bcl2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor 2
Bim	Bcl2 like 4
BM	Bone marrow

CRP	C-reactive protein
CSCs	Cardiac stem cells
CXCR4	CXC chemokine receptor 4
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-related kinase 1/2
ESCs	Embryonic stem cells
GFP	Green fluorescence protein
hESCs	Human embryonic stem cells
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
JAK	Janus kinase
LDL	Low-density lipoprotein
LVEF	Left ventricular ejection fraction
MEK	Mitogen-activated protein/extracellular signal-regulated kinase
miR	microRNA
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
MTPs	Microthrombotic particles
NO	Nitric oxide
OCN	Osteocalcin
OPN	Osteopontin
ROCK	Rho-associated coiled-coil-forming kinase
ROS	Reactive oxygen species
SDF-1α	Stromal cell-derived factor-1
SkMs	Skeletal myoblasts
SPECT	Single-photon emission computed tomography
STAT	Signal transducers and activators of transcription
STAT	Signal transducers and activators of transcription
STEMI	ST-elevation myocardial infarction
VEGF	Vascular endothelial growth factor

Introduction

One of the most promising areas in the current research and prediction for the future is in basic and translational research exploring the conceivable use of stem cells for cell-based and cell-free therapies. These unique cells can transform into and regenerate the different tissues that make up the human body. Moreover, they represent the basic building blocks of human development at various levels, whether therapeutic, convalescent, or others (Zakrzewski et al. 2019). Stem cells also release a plethora of bioactive molecules and lipid membrane-enclosed vesicles as part of their paracrine activity that also plays a significant role in the repair process (Haider and Aslam 2018). They are characterized by unlimited

undifferentiated self-renewal, clonogenicity, and transdifferentiation into different cell lineages with morphofunctional competence in response to different chemical cues and under a unique set of conditions. Hemopoietic stem cells (HSCs) were first recognized and displayed all these properties (Ng and Alexander 2017). Today, many types of tissue-specific resident stem/progenitor cells, i.e., bone marrow-derived mesenchymal stem cells (MSCs), adipose tissue derived stem cells, resident cardiac stem cells (CSCs), skeletal myoblasts (SkMs), umbilical cord blood-derived stem cells, peripheral blood-derived stem cells, etc., have been identified, isolated, and characterized from almost every body tissue (Bhartiya 2021). It is generally perceived that they are responsible for tissue repair and regeneration in the event of tissue injury. Incidentally, SkMs (Haider et al. 2004a), bone marrow-MSCs (Haider and Ashraf 2005), and CSCs (Leong et al. 2017) have already reached the clinical phase for safety and efficacy assessment in the human patients, and bone marrow-derived MSCs have advanced to phase III clinical trials (Rajab et al. 2019).

Essentially, stem cells are found in both embryonic and adult tissues, and hence, they are referred to as embryonic stem cells (ESCs) and adult tissue-derived stem cells, respectively. Developmental activity decreases the differentiation potential of stem cells with each step from pluripotency to terminal differentiation when the cells become specialized. Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) (Pera et al. 2000). They can be propagated in vitro indefinitely in the primitive undifferentiated state while retaining their pluripotency status (Vazin and Freed 2010). During embryonic development, ESCs form multiple assemblies with distinct functions called germ layers, which ultimately lead to the emergence of differentiated cells and tissues of the embryo, and later the adult organism (Larijani et al. 2012). A new entrant to this long list is the induced pluripotent stem cells (iPSCs), which are considered as surrogate ESCs albeit without the problem of ethical and moral issues and provide a renewable source for theranostic applications (Lalit et al. 2014; Ibrahim et al. 2016; Cagavi et al. 2018). Takahashi and Yamanaka first reported them in 2006, who successfully reprogrammed mouse tail fibroblasts using a quartet of pluripotency determining transcription factors, i.e., Oct3/4, Sox2, Klf4, and cMyc. Since then, iPSCs have been extensively studied for tissue regeneration in experimental animal models, including myocardial repair (Ahmed et al. 2011a; Buccini et al. 2012), however not without the threat of tumorigenicity (Ahmed et al. 2011b).

One of the most severe and widespread cardiovascular ailments is ischemic heart disease (IHD), which is now considered as the leading cause of morbidity and mortality globally. According to the figures released by the American Heart Association, one out of every three deaths reportedly occurred due to cardiovascular issues and incurring heavy financial burden (AHA 2018; WHO 2017). IHD leads to an irreversible impairment of cardiac function, which is generally attributed to the terminal differentiation status of the cardiomyocytes that renders them incapable of entering into the cell cycle in the event of injury (Rosenstrauch et al. 2005). Although the presence of resident CSCs has been reported (Frati et al. 2011; Mayfield et al. 2014), besides the ability of cardiomyocytes to reenter into the

proliferative cycle (Torella et al. 2015; Miyawaki et al. 2017), both these findings could contribute little in the intrinsic repair process, especially if the ischemic myocardial injury is extensive and involves massive myocyte injury and death. The contemporary treatment methods are limited to symptomatic relief without addressing the underlying cause and mechanistic interference in the pathological process, which continues as an unabated vicious cycle. The limitations of the current treatments have led to the search for novel treatment strategies aimed to regenerate and repair the damaged heart muscle so that the treatment extends over long-term and stable benefits. Stem cell therapy has given encouraging results in experimental animal models and clinical settings (Rajab et al. 2019).

Recent advances in stem cell biology and tissue engineering have given further impetus to regenerative medicine and its applications for myocardial repair. Despite these encouraging results, there are various aspects of stem cell-based therapy, which significantly hamper its progress to the clinical settings as a routine therapeutic modality. One of these impediments is the extensive death of the donor cells posttransplantation in the hostile microenvironment of the ischemic myocardium due to infiltrating inflammatory cells and a plethora of secreted proinflammatory cytokines, i.e., TNF-α, IL-1β, IL-6, etc., in response to the ischemic insult (Khodayari et al. 2019). This chapter focuses on the problem of extensive donor stem cell death-related issues and the preconditioning strategy to enhance donor cell survival. The particular focus of the chapter is on statins as preconditioning mimetics from the standpoint of pharmacological manipulation of the cells as well as the infarcted myocardium to render it conducive and favorable for the transplanted cells.

Stem Cell-Based Therapy for Myocardial Repair

Under the right physiological conditions and in response to the appropriate microenvironmental cues, stem cells divide and differentiate to form specialized cells with well-defined functions (Carbone et al. 2021). Given that ischemic myocardial damage is increasing the incidence of heart failure worldwide (Jenča et al. 2021) and the limited intrinsic regenerative capacity of the heart, cell-based therapy offers a potential therapeutic modality for routine use in the clinical settings. The published data has clearly shown the safety and feasibility of stem-cell-based therapy during the experimental phase and in the clinical trials (► Chap. 10, "Mesenchymal Stem Cells for Cardiac Repair").

Stem cells used for myocardial repair and regeneration have been derived from various tissue sources that include adult tissue-derived stem cells, i.e., bone marrow-derived MSCs, SkMs, resident CSCs, etc., embryo and embryo-associated tissue-derived stem cells, i.e., ESCs, umbilical cord blood-derived stem cells, etc., and iPSCs; each one with unique biological characteristics and stemness features. For example, the primary advantage of human ESCs over adult stem cells is their pluripotency. However, the moral and ethical issues ascribed to their use, tumorigenicity, and immunogenicity as well as arrhythmogenicity of the donor cell graft-derived cardiomyocytes have hampered their progress to the clinic. On the same

note, iPSCs are being assessed in translational experimental animal models for myocardial repair and regeneration (Li et al. 2013; Ye et al. 2014; Patrick et al. 2014). Contrarily, adult tissue-derived stem cells are easily accessible without ethical or moral issues, but with limited differentiation potential as compared to their pluripotent counterparts. Given their robust nature and autologous availability, three of them, including bone marrow-derived MSCs, mononuclear cells, and CSCs, have successfully progressed to the clinical assessment for myocardial repair (▶ Chap. 10, "Mesenchymal Stem Cells for Cardiac Repair").

Mesenchymal Stem Cells in Myocardial Repair and Regeneration

MSCs are self-renewing clonal precursors for nonhematopoietic tissues. They are characterized by preferential adherence to the plastic surface, expression of specific membrane markers, and trilineage differentiation potential (▶ Chap. 10, "Mesenchymal Stem Cells for Cardiac Repair"). Available data suggest that bone marrow-derived MSCs are suitable for the regeneration of infarcted myocardium. After decades of characterization in vitro, in small and translational experimental animal models, bone marrow-derived MSCs are the only cell type that has entered the advanced phases of clinical assessment. Hence, the fields of cell-based therapy, cell-free therapy (using conditioned medium and exosomes), and tissue engineering have primarily focused on MSCs owing to their wide range of properties, i.e., ease of availability and in vitro expansion, multilineage differentiation potential, paracrine activity, immunomodulatory and anti-inflammatory properties, etc. (Carson et al. 2018). However, the failure of the transplanted cells to thrive post engraftment in the infarcted myocardium has been generally ascribed to short-term beneficial effects of the cell therapy in most cases, both in the experimental settings and clinical trials, as further discussed in the next section.

Poor Survival of Stem Cells Postengraftment

Irrespective of the cell type, cell survival remains an issue in cell-based heart therapy (Li et al. 2021a). Like any other stem/progenitor cells, MSCs present a significant limitation post engraftment as they have a low survival rate after being transplanted in or around the infarcted myocardium, with the majority lasting for no more than 1 day (Penicka et al. 2005). Only a meager percentage of the transplanted cells survive for 3 months post-transplantation in some cases (Han et al. 2019). The massive death of the donor cells primarily occurs during early phase transplantation after ischemic injury. In one of the studies using [^{14}C]-thymidine-labeled cells, it was observed that only 44% of cells were present 10 min after intramyocardial injection, which decreased to 14% by 24 h and 7% after 72 h (Suzuki et al. 2004). Gyöngyösi et al. used a trifusion protein lentiviral transduction of porcine bone marrow MSCs to label them and study their fate after intramyocardial delivery. The trifusion protein included luciferase, red fluorescent protein (RPF), and herpes simplex thymidine kinase. PET-computed tomographic

metabolic and perfusion imaging was performed after intravenous injection of 10 mCi [18F]-FHBG, which showed decreased cardiac uptake of the delivered cells which was confirmed by luciferase activity as well. On day ten after injection, histological evidence showed 5.8% of the transplanted cells to survive in a porcine ischemic heart with a concomitant reduction in the infarct size (Gyöngyösi et al. 2008). On the same note, as little as 0.44% of the transplanted human MSCs were reported to survive by day 4 postengraftment in the experimental immune-deficient mice heart (Toma et al. 2002). The cells were labeled with *lac-z* reporter gene for tracking the fate of the cells posttransplantation. Similar results regarding the poor survivability of donor cells have also been reported in the clinical settings. For example, intracoronary infusion of ^{18}F-FDG-labeled MSCs showed meager 1.3–2.6% retention of the delivered cells after an hour of delivery (Hofmann et al. 2005).

As poor donor cell survival remains one of the primary limitations of cell-based therapy (Van Nguyen et al. 2021), the two primary mechanisms underlying donor cell death include the following: first, myocardial tissue turns inhospitable for the donor cells due to its highly acidic, hypoxic, cytokine-rich environment with scarcity of nutrients and high-level oxidative stress, and infiltration by the inflammatory and immune cells. Together, the molecular and cellular events in the infarcted myocardium present a significant challenge for transplanted stem cells, especially during the acute phase after ischemic injury (Bonvini et al. 2005; Chen et al. 2012). Second, the donor cells are not conditioned generally to withstand the rigors of the infarcted myocardium, in addition to anoiksis due to poor adhesion with the recipient's cardiac tissue during acute phase after transplantation (Zvibel et al. 2002), that lead the donor cells to undergo apoptosis (Tu et al. 2019).

Various strategies have been proposed to address the problem of massive loss of donor cells post engraftment. However, for the most part, these strategies have given dismal results. Nonetheless, preconditioning and reprogramming of cells by physical, chemical, genetic, and pharmacological manipulations have given encouraging data at least in the experimental settings to be pursued for clinical applications in the future (Haider and Ashraf 2012). Each preconditioning strategy primes the cells to withstand the severity associated with fatal ischemia in vitro and posttransplant (Wei et al. 2017).

Strategies to Enhance Donor Cell Survival

Stem Cell Preconditioning

Preconditioning refers to the protection of tissues and cells from injury when subjected to a subsequent innocuous environment. Preconditioning offers the most potent known cytoprotective effects in the physiological systems. Since the pioneering work of Charles Murry, conditioning from cyclical exposure to periodic ischemia-reperfusion sublethal episodes is cardioprotective via slowing down the rate of ATP depletion during ischemia and washing out of the catabolites during reperfusion (Murry et al. 1986). This strategy has been extrapolated to maintain its beneficial effects at the cellular and subcellular levels (Lu et al. 2010; Gu et al. 2021).

The underlying rationale of transient exposure to sublethal episodes of a noxious stimulus triggers a survival-signaling pathway that renders cells resistant to a subsequent lethal stimulus. More recently, the preconditioning strategy has been successfully extrapolated to condition stem cells to enhance their stemness properties in general and post engraftment survival in particular (Haider and Ashraf 2012).

Besides the classical ischemia-reperfusion-based treatment protocol, various pretreatment strategies have been developed and optimized to prime the cells into a "state of readiness" to combat chronic lethal exposure, which includes physical, chemical, genetic, and pharmacological manipulation of cells to mimic effects of ischemic pretreatment (Haider and Ashraf 2010). Haider et al. have reported the novel strategy of ischemic preconditioning of bone marrow-derived (MSCs) using short intermittent cyclical exposure to anoxia/reoxygenation (Kim et al. 2009). Elucidating the underlying mechanism, they have shown that their ischemic preconditioning protocol activated Akt(ser473) and Erk1/2(Thr202/Tyr204) with a consequent HIF-1α nuclear translocation and a consequent involvement of hypoxamir-210. Furthermore, real-time PCR array for rat apoptotic genes, computational target gene analyses, and luciferase reporter assay identified FLICE-associated huge protein (FLASH)/caspase-8-associated protein-2 (Casp8ap2) suppression in the preconditioned cells as the downstream target gene of miR-210 that was involved in cytoprotection. A subsequent experimental study by Haider et al. also showed a significant role of hypoxamir-107 in cytoprotection, and its simultaneous induction or concomitant transgenic overexpression with miR-210 in MSCs could mimic the cytoprotective effects of ischemic preconditioning (Kim et al. 2012a). The authors also observed that MSCs overexpressing miR-210 had higher survival postengraftment in the infarcted myocardium in the experimental rat model and contributed to the angiomyogenic repair of the myocardium (Kim et al. 2012b). Encouraged by the data, the authors have reported the development and optimization for preconditioning of stem cells via subcellular mechanism (Lu et al. 2010, 2012b). Some of the other strategies reported by the same group of researchers include pharmacological treatment, i.e., diazoxide, tadalafil, etc., (Niagara et al. 2007; Afzal et al. 2010; Haider et al. 2010; Suzuki et al. 2010), treatment with growth factors (Elmadbouh et al. 2011; Lu et al. 2012a), genetic modification of cells for growth factor overexpression, IGF-1, Netrin, etc., (Haider et al. 2008; Durrani et al. 2012), or concomitant transgenic overexpression of angio-competent growth factor and survival-signaling molecules, i.e., angiopoietin-1 and Akt, etc., (Jiang et al. 2006), treatment with small molecules, i.e., nicorandil, sodium butyrate, etc., (Tabeshmehr et al. 2017; Hosseini et al. 2018), and lysate from pharmacologically preconditioned cells (Afzal et al. 2008).

Augmenting the Myocardial Microenvironment Favorable for Donor Cells

The interaction between the microenvironment of the infarcted myocardium and the transplanted cells is one of the primary determinants of the outcome of myocardial cell therapy (Farhudg et al. 2019). In the case of MSCs, although MSCs have potent

anti-inflammatory and immunomodulatory effects, augmentation of the hostile myocardial microenvironment to make it favorable for receiving the donor MSCs postengraftment has been studied as an alternative approach to the donor stem cell preconditioning (Ezquer et al. 2017). In this regard, various strategies based on physical, chemical, or pharmacological pretreatment of the heart have been explored. For example, transient immunosuppression starting a few days before cell therapy results in significantly enhancing survival and engraftment of the transplanted cells in the infarcted heart (Xiao et al. 2004; Haider et al. 2004b). Similarly, during concomitant administration of cells with growth factor treatment, the latter helped as a modifier of the local microenvironment of the myocardium for the transplanted cells (Li et al. 2014). However, given the short biological half-life of the immunomodulating and tissue-modifying growth factors, the use of biomaterials loaded with growth factors and cytokines together with cell therapy is gaining popularity (Smagul et al. 2020). Another approach adopted during cell therapy has been to genetically modify the cells to secrete a cocktail of growth factors, which besides initiating survival signaling in the cells also provide a source of extended release of growth factors that favorably modify the microenvironment of the cell in the infarcted heart (Konoplyannikov et al. 2013).

Besides, genetic modification and growth factor treatment-based combinatorial approaches, some protocols are based on physical manipulation of the myocardial microenvironment to enhance its receptivity for the donor cells. For example, the cell-wave therapy approach has been effectively applied to the experimental animal models of MI and myocardial ischemia (Gollmann-Tepekoylu et al. 2018; Holfeld et al. 2016). After successful and encouraging data, it has progressed to safety and feasibility assessment in clinical trials (Assmus et al. 2013). The underlying mechanism has been attributed to the induction of toll-like receptor signaling, increased cell proliferation, increased level of VEGF, and mobilization and homing-in of endothelial cells besides cardioprotection (Aicher et al. 2006, Tepekoylu et al. 2013; Holfeld et al. 2014, 2016). Recent advancements in physical manipulation include ultrasound target bubble destruction (UTBD) to enhance vascular perforation and extravasation of the cells.

Treatment with statins in this regard has emerged as an approach that could simultaneously prime the cells and enhance the receptivity of the infarcted myocardium for donor cells.

Statins as Preconditioning Mimetic and Modifier of Myocardial Microenvironment

Introduction to Statins

Developed in 1987, statins are pharmacological agents that act as β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (Endo 2010). The first statin to receive approval for clinical use was lovastatin, which naturally occurs in low concentrations in oyster mushrooms, red yeast rice, and other foodstuffs (Ferri and Corsini 2020). Over the years, a wide range of statins have been synthesized,

including atorvastatin, simvastatin, pravastatin, rosuvastatin, fluvastatin, and pitavastatin, each one with unique pharmacological characteristics but a common mechanism of action (Althanoon et al. 2020). Statins are designed to prevent three major categories of health conditions. First of all, they have been successfully used to attenuate the likelihood of cardiovascular diseases, including heart attack and stroke, especially in at-risk individuals older than 40 years. They are considered primary preventive treatment (Zeiser 2018). Second, statins lower the probability of further cardiovascular conditions in individuals suffering from cardiovascular disease referred to as secondary prevention. Finally, statins can prevent the development of hyperlipidemia by decreasing excessively high levels of blood cholesterol (Xu et al. 2013). There is a correlation between heightened cholesterol levels and cardiovascular disease. As such, statins are intended to diminish the likelihood of heart attack and stroke in the long term by reducing dangerously high levels of low-density lipoprotein (LDL (cholesterol (Ferri and Corsini 2020).

Functional Mechanism of Statins

Statins work by suppressing the enzyme HMG-CoA reductase, which functions as a rate-limiting enzyme within the mevalonate pathway associated with the production of cholesterol. Statins lower the blood LDL levels through competitive inhibition of HMG-CoA reductase (Stroes 2005). Another role fulfilled by statins is to decrease the amount of cholesterol that is produced by the liver. Internal physiological processes are the primary source of cholesterol in the circulation. The level of cholesterol in the blood declines due to statin-induced reduction of cholesterol produced by the liver (Young and Fong 2012; Zeiser 2018). The decrease in the levels of cholesterol triggers two events: LDL receptors are produced more extensively by liver cells to enable extraction of cholesterol from the bloodstream, and second, lipoproteins of low and extremely low density (the so-called “bad cholesterol”) undergo extraction and eventual conversion to bile acids and additional by-products (Han et al. 2019). Therefore, the function of statins is not limited to decreasing the levels of LDL cholesterol; instead, it extends more than that (Andrzejewska et al. 2019).

There is mounting evidence in the published data that statins contribute to endothelial cohesion and modulate inflammatory processes that can cause atheroma to develop. Moreover, statins keep plaque stable and make it less likely for blood clots to form (Xu et al. 2013). Additionally, statins regulate the proliferation, migration, and survival of tumor cells by regulating Rho, Ras, and Rac proteins. Further, they can also inhibit cancer cell growth by modulating specific other pathways.

The Impact of Statins on Mesenchymal Stem Cells

According to the published data, statins significantly enhance the outcome of cell-based therapy using MSCs. Studies on cardiac tissue engineering and myocardial

infarction therapy have reported that treatment strategies integrating statins and MSCs helped the heart function better by enhancing the LVEF and end-systolic pressure of the left ventricle while also contributing to myocardial repair by promoting cardiomyocyte protection and proliferation at the site of myocardial injury (Zeiser 2018; Cianflone et al. 2020). In vitro studies have also shown that treatment of CSCs with rosuvastatin, simvastatin, and pravastatin significantly enhances their proliferation and myocardial differentiation (Cianflone et al. 2020). These data have prompted nanoparticle-based and polymer-based delivery of statins to manage cardiovascular diseases (Nenna et al. 2021). Meanwhile, it has been proven that atorvastatin can ameliorate scar hypertrophy by reducing the thickness of the dermis and epidermis and by intensifying the production of the prorepair cytokine C-X-C motif chemokine ligand 8 (CXCL8) from MSCs of adipose origin in skin counterparts (Andrzejewska et al. 2019).

Studies on cardiac tissue engineering and myocardial infarction therapy have reported that treatment strategies integrating statin administration with MSCs-based therapy helped the global heart function to improve by enhancing LVEF and LV end-systolic pressure while also contributing to the cardiac repair mechanisms by promoting cardiomyocytes proliferation at the site of myocardial injury (Zeiser 2018) and attenuate myocardial hypertrophy (Nakagami and Liao 2004).

The potential of MSCs to differentiate appears to be affected by statins (Zeiser 2018). This conclusion is supported by observations that MSCs' osteogenic differentiation was encouraged by the release of simvastatin from poly (lactic-co-glycolic acid) (PLGA) nanoparticles integrated into bone substitute based on commercial calcium phosphate (Chamani et al. 2021). Overall, the proteins related to MSC osteogenic differentiation, including core-binding factor α1 (CBFA1), HIF-1α, and bone sialoprotein, are highly expressed in the presence of statins (Potier et al. 2007; James 2013). Furthermore, statins also enhance osteocalcin (OCN) gene expression, which reflects terminal differentiation of osteoblasts, and osteopontin (OPN), which is a sialoprotein with a high degree of phosphorylation present in the mineralized extracellular matrix (ECM) of bones and teeth and participates during the late phase osteoblast differentiation (Zurick et al. 2013). Moreover, some statins intensify the action of the osteogenic marker called alkaline phosphatase (ALP) (Xue et al. 2019; Chapman and Tanner 2021) and the expression of collagen type-Iα 1 (COL1A1) (Ruiz-Gaspa et al. 2007), which is vital for the development of ECM and osteogenic differentiation and is a marker of osteoblast maturation.

Statins may double bone morphogenic protein-2 (BMP-2) levels, which induces osteoblasts to differentiate (Alam et al. 2009). BMP-2 has a very short half-life; hence it is impractical to add it to the differentiation media in culture, which is why it is highly significant that MSCs are primed to produce BMP-2 in greater quantities to make it cost-effective and achieve sustained presence in the culture. In some cases, statin treatment has been combined with BMP-2 treatment to enhance osteoprecursor cells' differentiation (Park 2012). Furthermore, MSCs have elevated expression of Runt-related transcription factor 2 (RUNX2) in the context of simvastatin treatment (Chen et al. 2010). The discharge of simvastatin from TiO2 nanotubes has also been

shown to promote osteoblast differentiation and inhibit osteoclast resorption (Lai et al. 2017).

Statins and Stem Cell Preconditioning

Statins and In Vitro Experimental Studies

MSCs are deficient in nutrients and oxygen during ex vivo expansion. Though feasible, the supplementation of trophic factors in the culture medium is insufficient to support their stem cell functions optimally. Moreover, MSCs isolated from unhealthy and aging individuals or those maintained over an extended period undergo apoptosis and senescence. Besides other factors, these changes are caused by an imbalance between ROS generation and antioxidant mechanisms in the cells, leading to the inactivation of cytoprotective NO production and apoptosis.

Treatment of stem cells with statins as preconditioning mimetics has been extensively studied during in vitro experimental studies to exploit their ability to abrogate ROS activity and enhance NO activity (Wassmann et al. 2002). For example, Dong et al. (2011) have shown the cytoprotective effects of atorvastatin treatment on porcine BM-derived MSCs cultured under serum-free and hypoxic culture conditions (Dong et al. 2011). Molecular studies revealed that atorvastatin pretreatment significantly enhanced antiapoptotic protein Bcl2 expression in the cells besides higher level phosphorylation of AMPK and eNOS, which was abrogated in the presence of compound C, an inhibitor of AMPK. A similar molecular mechanism of atorvastatin-induced cytoprotection in MSCs has also been reported by several other research groups (Sun et al. 2006; Song et al. 2011; Zhang et al. 2012). Lovastatin also protects MSCs against hypoxia/serum deprivation-induced apoptosis, focusing on the mitochondrial pathway (Xu et al. 2008). Preconditioning with lovastatin (0.01–1 uM) significantly abrogated cytochrome c release from mitochondria, thus preventing the executioner caspase 3 activation. The cytoprotective effects of lovastatin preconditioning were abrogated by PI3K inhibitor LY294002, which inhibited ERK1/2 activation. These data showed the significant involvement of PI3K/Akt and MEK/ERK1/2 signaling in the cytoprotective effects of lovastatin preconditioning of MSCs. Zhang et al. have reported the RhoA/ Rho-associated coiled-coil-forming kinase (ROCK)/ERK pathway role in protecting cells against hypoxia and serum deprivation in culture conditions (Zhang et al. 2014). Treatment with atorvastatin enhanced cell survival, which was mimicked by ROCK inhibitor fasudil. The involvement of RhoA/ROCK in cardiomyocyte protection has also been reported after fluvastatin treatment (Yi et al. 2020).

Izadpanah et al. studied the impact of statin treatment on MSCs' biological characteristics (Izadpanah et al. 2015). They treated ex vivo cultured MSCs with atorvastatin and pravastatin at clinically relevant concentrations and studied their rate of proliferation and differentiation potential. After statin treatment, the authors observed significantly increased doubling-time and colony-forming units in both young and old donor adipose tissue-derived MSCs. Moreover, statin treatment also

reduced MSCs' osteogenic and chondrogenic differentiation potential besides their macrophage differentiation. Molecular studies revealed upregulated expression of p16, p53, caspase 3, 6, and 9 in the statin-treated MSCs.

A recently published study by Nantavisai et al. has reported that simvastatin treatment enhanced the expression of pluripotency markers in canine bone marrow-derived MSCs besides increasing their proliferation (Nantavisai et al. 2019). Treating the cells with four different doses of simvastatin ranging from 0.1 nM to 100 nM, the authors observed a dose-dependent increase in the rate of cell proliferation on days five and seven after treatment. Molecular studies revealed increased cyclin D1 and D2, Ki67 and antiapoptotic protein Bcl2, and reduced caspase-8 and -9 expressions at higher doses. Low-dose treatment increased the propensity of Rex1 and Oct4 expression.

Statins and in vivo Experimental Animal Models

A systematic review and meta-analysis of 5 studies regarding the combined efficacy of atorvastatin and MSCs-based cell therapy for treating acute myocardial infarction (AMI) in experimental animal models clearly shows the superiority of the combinatorial therapeutic approach over either treatment alone (Dai et al. 2015). The pooled analysis of the studies showed a significantly improved LVEF in the combined treatment group than MSCs-treated group without atorvastatin. The systematic review concludes that combining MSCs-based cell with atorvastatin-based pharmacotherapy is a more effective therapeutic approach for further assessment in the clinical settings. On the same note, a systematic review of 38 studies, including 18 preclinical studies involving statin-treated MSCs-based cell therapy, has reported significantly improved biology and stemness characteristics of stem/ progenitor cells. The combined treatment approach using preconditioned MSCs with statin therapy led to improved organ function (Park et al. 2016).

Combined Statin and MSCs-Based Cell Therapy

Using a mini-swine experimental model of acute myocardial infarction, Dong et al. reported significant enhancement of the therapeutic efficacy of combining cell-based therapy with daily atorvastatin treatment (0.25 mg/kg) after intramyocardial delivery of the cells, with or without L-NNA treatment, an inhibitor of atorvastatin (5 mg/kg/day) (Song et al. 2013). Interestingly, atorvastatin treatment significantly enhanced the donor cell survival postengraftment. Single-photon emission-computed tomography (SPECT) revealed reduced areas of the perfusion defect. At the same time, MRI showed significantly improved LVEF 4 weeks after treatment with a concomitant reduction in infarct size, the extent of fibrosis, and the rate of apoptotic cells in the combined atorvastatin and MSCs-treated animals as compared to the control animal group. The authors attributed the beneficial effects to improved donor cell survival, reduction in inflammatory markers, i.e., CRP, and improved paracrine activity of MSCs in the presence of atorvastatin. Similar data have also been reported when simvastatin was administered after MSCs-based cell therapy as a part of the

combinatorial approach (Yang et al. 2009). At 6 weeks, MRI revealed significantly attenuated infarct size and the number of dyskinetic segments. Treatment with simvastatin also reduced the number of apoptotic cells in the myocardium.

Treatment with atorvastatin has been reported to accentuate SDF-1α expression in the infarcted myocardium via nitric oxide production, which is generally increased in response to ischemic injury (Qiu et al. 2012). Tian and colleagues have reported atorvastatin-induced activation of the SDF-1α/CXCR4 axis as the possible underlying mechanism for their observed improvement in cardiac function in a rodent model of acute myocardial infarction (Tian et al. 2019). The authors exploited the elevated SDF-1α release from cardiomyocytes in response to ischemic injury and induction of CXCR4 on MSCs in response to atorvastatin preconditioning. Transplantation of atorvastatin-preconditioned MSCs significantly reduced scar formation and improved indices of myocardial function at 4 weeks after treatment. Interestingly, transplantation of preconditioned MSCs combined with oral administration of atorvastatin (10 mg/kg/day) potentiated the efficacy of preconditioned cells which was blocked by AMD3100 (SDF-1/CXCR4 specific antagonist). The same research group has also reported enhanced expression of CXCR4 on endothelial cells and increased emigrational activity of MSCs in a rat heart model of ischemia-reperfusion injury (Chiang et al. 2015; Cai et al. 2013).

Li et al. 2015 have also reported increased CXCR4 expression in atorvastatin-preconditioned MSCs within 24 h after preconditioning as evidenced by flow cytometry and real-time PCR and confirmed by a transwell system, which revealed their enhanced emigrational activity in response to SDF-1α gradient (Li et al. 2015). Intravenously delivered atorvastatin-primed MSCs homed-in to the infarcted rat heart in significantly higher number than the naïve (nonpreconditioned) MSCs and showed significantly higher survival on day 30 after transplantation. Moreover, treatment with preconditioned MSCs preserved the infarcted heart function and significantly reduced fibrotic tissue in the infarcted rat heart. While elucidating the molecular mechanism underlying the improved emigrational activity of atorvastatin-treated MSCs, it was observed that the induction of the CXCR4 expression was regulated by miR-146a, thus establishing miR-146a as a potential target to modulate MSCs' emigrational activity (Li et al. 2021b).

Rosuvastatin is considered a "super statin" due to many of pharmacological functions, including strong anti-inflammatory and antioxidant properties. Akin to atorvastatin, rosuvastatin treatment has been reported to enhance MSCs survival postengraftment in the infarcted heart (Fu et al. 2016; Zhang et al. 2013). Fu et al. hypothesized that combining rosuvastatin with MSCs transplantation would enhance the survival of transplanted cells via attenuation of inflammatory response in the infarcted myocardium (Fu et al. 2016). Using an experimental rat model of coronary microembolization, using microthrombotic particles (MTPs), GFP-labeled allogenic MSCs were delivered by intracardiac injection (into the left ventricle) simultaneously with MTPs. Rosuvastatin was administered 7 days before the cell transplantation and continued until 7 days after cell delivery. The authors observed significant reduction in proinflammatory cytokines, i.e., TNF-α, IL-1β, as well as infiltration of inflammatory cells. Rosuvastatin treatment significantly enhanced the

transplanted MSCs' survival (~45 fold) with concomitant increase in proangiogenic factors, i.e., VEGF and bFGF. In another study combining rosuvastatin (10^{-6} mmol/L) treatment with MSCs engraftment, Zhang et al. used murine MSCs expressing luciferase and GFP (Zhang et al. 2013). Rosuvastatin treatment significantly enhanced transplanted cells' viability and reduced cardiomyocyte apoptosis rate in the infarcted myocardium with a significant reduction in the area of fibrosis. Molecular studies showed enhanced Akt and ERK activation and reduction in proapoptotic proteins, including Bim and Bax. Another important observation was improved paracrine activity of the transplanted cells, which were counted as an integral part of the underlying mechanism leading to the overall reduction in apoptosis and improved cardiac function.

The Janus kinase (JAK /Signal transducers and activators of transcription (STAT) signaling is another essential pathway activated during statin treatment of MSCs and majorly impacted the efficacy of preconditioned cells (Xu et al. 2011). JAK/STAT is well documented for its role in cell stress response and cardioprotection (Kurdi and Booz 2009; Kiu and Nicholson 2012). The combinatorial treatment approach involving MSCs-based cell therapy and concomitant treatment with rosuvastatin provides an alternative approach that helps to render the cardiac microenvironment conducive for the transplanted cells rather than directly preconditioning the cells. Xu et al. have reported significantly improved survival, engraftment, and differentiation of the sex-mismatched transplanted MSCs in an infarcted female rodent heart model (Xu et al. 2011). Starting 1 day before intramyocardial injection of $2x10^6$ MSCs, rosuvastatin administration (4 mg/kg/day) was continued until 5 weeks with or without AG-490 treatment (JAK/STAT inhibitor, CalBiochem). The authors provided histological evidence of significant angiomyogenic activity in and around the infarcted myocardium, clearly abrogated by AG-490. There was a substantial improvement in cardiac function indices, including LVEF and LVFS, and reduction in the fibrotic area at 4 weeks after combined MSCs and statin treatment. Western blot analysis showed accentuation of JAK-STAT phosphorylation in rosuvastatin treatment animal hearts. More specifically, phospho (p)JAK2/JAK2, pSTAT1/STAT, and pSTAT3/STAT3 were significantly higher in the combined treatment group than the MSCs alone treated animal hearts. Another significant signaling pathway reported in cytoprotection in response to atorvastatin treatment is RhoA/ Rho-associated coiled-coil-forming kinase (ROCK), reported in experimental female heart model with AMI after sex-mismatched MSCs transplantation (Zhang et al. 2014). The cells showed significantly enhanced survival but with little evidence of transdifferentiation. Moreover, atorvastatin treatment has significant immunomodulatory effects with a pronounced reduction in proinflammatory cytokines.

In summary, these studies provide mounting evidence that combined treatment with statin supported the reparability of the transplanted MSCs by rendering the myocardial milieu conducive for their survival and functioning. For example, at molecular and subcellular level, it has been reported that simvastatin and atorvastatin attenuate collagen and collagen-associated proteins expression in the infarcted myocardium, while simvastatin promotes exosome release from the cardiomyocytes,

which are rich in decorin and deficient in periostin contents (Zeng et al. 2019; Kuo et al. 2019).

Using Statin-Preconditioned MSCs to Treat AMI

For optimal therapeutic outcome, Xu et al. combined atorvastatin-preconditioned MSCs with atorvastatin therapy at three different time points after AMI and delivered one to three cell injections (Xu et al. 2019a). In their extensive experimentation divided into two parts, the authors first determined the time of peak level SDF-1α expression in the infarcted heart post ischemic episode. Based on their findings, the second half of the experiment was designed to assess the time of intravenous delivery of atorvastatin-preconditioned MSCs at early, mid, and late time-point delivery of single, double, and triple doses of the cells with or without intensive treatment with atorvastatin. A total of 215 female rats were randomized in 11 different treatment groups. Although therapeutic benefits were observed in all the cell treatment groups of animals, the highest therapeutic benefits were achieved in the late-treatment group of animals with triple injections, which showed significantly higher homing of the delivered cells into the infarcted myocardium as compared to the other treatment groups. These animals also had significantly reduced infarct size, attenuated fibrosis, and improved cardiac function; there was little evidence of cardiomyogenic differentiation of the preconditioned MSCs. Besides showing the therapeutic significance of the statin-based preconditioning approach, these data also showed the importance of a multiple cell delivery strategy.

The use of MSCs-derived exosomes is gaining popularity for the treatment of injured myocardium as it alleviates the unwanted effects of cell-based therapy (Haider and Aramini 2020; Haider and Aslam 2018). A recent advancement in the atorvastatin-based combinatorial approach is based on the use of atorvastatin and MSCs-derived exosomes as part of the fast-emerging cell-free therapy for the treatment of ischemic stroke for neurological recovery (Safakheil and Safakheil 2020).

A summary of the various experimental animal studies involving the use of atorvastatin preconditioned MSCs or MSCs-based cell therapy combined with atorvastatin treatment is given in Table 1.

Statin-Based Preconditioning of MSCs and Exosomes

Preconditioned stem/progenitor cells release exosomes with enormous therapeutic potential as compared to the exosomes derived from naïve MSCs (Panda et al. 2021). For example, atorvastatin-preconditioned MSCs-derived exosomes had significantly higher proangiogenic potential for diabetic wound healing than exosomes derived from naïve (nonpreconditioned) MSCs (Yu et al. 2020). Treatment of human umbilical vein endothelial cells (HUVEC) in vitro with atorvastatin-preconditioned MSCs-derived exosomes significantly enhanced their emigrational potential, tube formation, and VEGF expression with the possible involvement of Akt/eNOS signaling and downstream upregulation of miR-221-3p.

Besides other effects, priming the MSCs with atorvastatin prompted the cells to release exosomes with significantly higher cardioprotective effects than the

Table 1 Summary of the important experimental animal studies reported using MSCs-based cell therapy combined with statin treatment as a combinatorial approach or for preconditioning of MSCs before transplantation

Animal model	Cells and route of delivery	Statin used	Summary of results	Reference
Swine experimental model of AMI	3x 10^7 cells by intramyocardial injection of MSCs	Atorvastatin 0.25 mg/kg/day	The intramyocardial delivery of MSCs was carried out in a mini swine heart model of AMI immediately after reperfusion The animals were pretreated without or with atorvastatin 30 days later, significantly higher survival of transplanted cells was observed in the atorvastatin-pretreated animals. MRI and SPECT showed significantly improved cardiac function and myocardial perfusion. Also, combined treatment reduced cardiomyocyte apoptosis, oxidative stress, and the suppression of proinflammatory cytokines in the infarcted myocardium	Yang et al. 2008
Rat-experimental model of AMI	1x 10^6 cells by intramyocardial injection	Atorvastatin 10 mg/kg/day	MSCs were cocultured with cardiomyocytes in transwell system and showed GATA4 and cTn-I expression and were used for transplantation study using non-cocultured MSCs as a control. Atorvastatin or vehicle was administered starting 24 h after AMI Four week later, the rate of survival of the cells and differentiation	Cai et al. 2011

(continued)

Table 1 (continued)

Animal model	Cells and route of delivery	Statin used	Summary of results	Reference
			was significantly higher in atorvastatin-treated animals. Also, significantly reduced inflammatory cytokines (i.e., TNF, VCAM, and CRP), myeloperoxidase activity, and infiltrating cells were observed by day 28 after treatment	
Rabbit experimental model of AMI	4x107 cells by intramyocardial injection	Atorvastatin 1.5 mg/kg/day	Thirty rabbits equally divided to receive no treatment, MSCs, and combined MSCs with atorvastatin. The combined treatment acted synergistically to improve LVEF, decreased LV-systolic and diastolic dimensions, reduced area of fibrosis, and reduced the inflammatory cell infiltration into the infarct zone There was significant improvement in MSCs survival as well as reduction in cardiomyocyte apoptosis	Qu et al. 2013
Rat model of chronic MI (7 days after MI)	2x 10^6 sex-mismatched MSCs by intramyocardial injection	Combined MSCs and Rosuvastatin 4 mg/kg/day	Significantly higher level survival of sex-mismatched MSCs in the infarcted heart which showed higher level cardiogenic differentiation as well. There was significantly higher angiogenic response Molecular studies revealed JAK/STAT pathway activation which was abrogated	Xu et al. 2011

(continued)

Table 1 (continued)

Animal model	Cells and route of delivery	Statin used	Summary of results	Reference
			by AG-490. There was significant reduction in cardiomyocyte apoptosis, reduction in fibrosis, and better functional improvement	
Swine experimental model of AMI	3x 10^7 cells by intramyocardial injection	Atorvastatin 0.25 mg/kg/day	Forty-two swines with AMI were grouped to receive sham, AMI control, MSCs, MSCs+ statin, and MSCs+statin+L-NNA. Four weeks after treatment, decreased perfusion defect areas on SPECT and metabolism by PET, significantly increased LVEF by MRI, and reduced apoptosis and area of fibrosis. The benefits were partially blocked by L-NNA which showed the involvement of eNOS activation	Song et al. 2013
Rat heart model of coronary microembolization	2x 10^6 GFP-labeled MSCs by intramyocardial injection	Rosuvastatin 3 mg/kg/day starting 7 days earlier until 7 days after cell treatment	Rosuvastatin treatment significantly reduced proinflammatory cytokines, i.e., TNF-a and IL-1b in the embolized myocardium, besides reduced infiltration of inflammatory cells Transplanted cell survival was significantly higher in rosuvastatin-treated animal hearts. Proangiogenic growth factors, i.e., VEGF and bFGF, increased significantly leading	Fu et al. 2016

(continued)

Table 1 (continued)

Animal model	Cells and route of delivery	Statin used	Summary of results	Reference
			to enhanced angiogenic response	
Rat model of experimental AMI	$5x10^6$ sex-mismatched MSCs by intramyocardial injection	Atorvastatin 10 mg/kg/day	Atorvastatin treatment combined with male donor MSCs transplantation in female recipient infarcted heart. Significantly higher survival of the donor cells; however, no trans differentiation was observed in the transplanted cells. However, significantly reduced proinflammatory cytokines with concomitant reduction in ROCK and ERK activities	Zhang et al. 2014
Rat model of experimental AMI	$2x10^6$ MSCs via tail vein injection	Combined atorvastatin 10 mg/kg/day or preconditioned MSCs	Atorvastatin pretreatment of MSCs significantly enhanced CXCR4 expression on the cells which enhanced their migration in response to SDF-1α in transwell system. Intravenous delivered preconditioned MSCs in a rodent heart model of AMI successfully homed-in to the infarcted myocardium, leading to improved cardiac function parameters as compared to the control animals and attenuated LV remodeling. The study proved the significance of SDF-1α/CXCR4 axis for homing and engraftment of	Li et al. 2015

(continued)

Table 1 (continued)

Animal model	Cells and route of delivery	Statin used	Summary of results	Reference
			intravenously delivered MSCs	
Rat model of experimental AMI	$2x10^6$ MSCs by intramyocardial injection	Combined atorvastatin 10 mg/kg/day or preconditioned MSCs	115 female rats with AMI divided into six groups of treatment (n = 15 per group). Atorvastatin preconditioned MSCs combined with atorvastatin therapy showed significant emigrational activity via activation of SDF-1α/CXCR4 signaling, the cells homed-in to the infarcted heart. Significant improvement in cardiac function parameters besides reduction in infarct size. The therapeutic benefits were abrogated by AMD3100 treatment	Tian et al. 2019
Rat model of experimental MI	$2x10^6$ MSCs via tail vein injection	Combined atorvastatin 10 mg/kg/day or preconditioned MSCs	First part of the experiment determined the time point at which SDF-1α level was maximum in the heart after MI (in 150 rats). In the second part, 215 female rats were randomized into 11 treatment groups ($n = 15$ rats per group) receiving preconditioned cells early, mid, and late time points using single dose, two doses, or three doses. All cell therapy groups showed significant homing-in of the transplanted cells; however, the	Xu et al. 2019a

(continued)

Table 1 (continued)

Animal model	Cells and route of delivery	Statin used	Summary of results	Reference
			highest cell number was achieved in late treatment group with triple injections. This group also showed highest angiogenesis when combined with atorvastatin treatment, with significantly reduced infarct size and area of fibrosis. There was little evidence of myogenic differentiation of the preconditioned cells	
Rat model of experimental AMI	Exosome derived from atorvastatin-preconditioned MSCs		Treatment with exosomes promoted significantly higher angiogenesis in the infarcted myocardium. There was significant reduction in proinflammatory cytokines IL6 and TNF in the infarcted myocardium. The exosomes derived from preconditioned MSCs were rich in lncRNA H19 to regulate miR-675 and activation of VEGF and intracellular adhesion molecule-1. Moreover, there was significant reduction in cardiomyocyte apoptosis in and around the area of infarct	Huang et al. 2020

AMI, Acute myocardial infarction; ERK1/2, Extracellular regulated protein kinase; L-NNA, NG-nitrol-L-arginine; LV, Left ventricle; LVEF, Left ventricular ejection fraction; MRI, Magnetic resonance imaging; PET, Positron Emission Tomography; ROCK, Rho-associated coiled-coil-forming kinase; SDF-1α, Stromal cell-derived factor-1α; SPECT, Single-Photon Emission-Computed Tomography; VEGF, Vascular endothelial growth factor; lncRNA, Long noncoding RNA

exosomes derived from naïve MSCs without atorvastatin treatment (Huang et al. 2020). Exosomal lncRNA sequencing of atorvastatin-primed MSCs showed nearly 450 lncRNA with upregulated expression (more than 1.5-fold increases) compared to the exosomes from naïve MSCs. Exosomal lncRNA H19 was increased almost 13-fold and had significant participation in angiogenesis and cardiac protection. Treatment of experimentally infarcted rodent heart with atorvastatin-primed MSCs-derived exosomes was cardioprotective, reduced cardiomyocyte apoptosis, increased angiogenesis, and reduced proinflammatory IL6 and TNF expression in the infarcted myocardium, which was attributed to the immunomodulatory effects of statins.

Although statin treatment enhances the therapeutic efficacy of the stem cell-derived exosomes, pretreatment of MSCs has been shown to abrogate exosome generation by the stem cells. For example, pretreatment of bone marrow-derived MSCs with simvastatin significantly reduces exosome biogenesis by depleting the synthesis of proteins needed for the generation of exosome (Kulshreshtha et al. 2019).

In vitro studies with atorvastatin-primed MSCs-derived exosomes showed significantly enhanced endothelial cell survival, emigrational potential, and tubulogenesis.

Combined Statin and Cell-Based Therapy for AMI Patients

Based on the encouraging data from small experimental animal models and translational studies (Table 1), and cardioprotective effects of high-dose atorvastatin treatment in the clinical settings (Teshima et al. 2009; Kim et al. 2010; Barbarash et al. 2015), combined statin and cell-based therapy has progressed to the clinical phase to treat patient with myocardial infarction. Xu et al. have designed TEAM-AMI (ClinicalTrials.gov Identifier: NCT03047772), a randomized double-blind placebo-controlled multicenter clinical trial of transplantation efficacy of autologous bone marrow mesenchymal stem cells with intensive atorvastatin in acute myocardial infarction patients (Xu et al. 2019b). The study was designed to enhance the efficacy of MSCs via combined therapy with atorvastatin by promoting their survival post engraftment in patients with anterior St-segment elevation MI. The patients were randomized to receive either routine atorvastatin therapy (20 mg/day) with placebo or intravenous injection of MSCs, or 80 mg/day or placebo (1:1:1:1). The primary end point of the study was absolute change in LVEF while the secondary end points were global cardiac function, remodeling, and regeneration. The study is anticipated to enroll 124 patients, and no data from it has been published as yet although the study was to be completed in 2018.

The same research group has registered another similar study STEM-AMI with an official title "Strengthening transplantation effects of bone marrow mononuclear cells with atorvastatin in myocardial infarction" (ClinicalTrials.gov Identifier:

NCT00979758) (Yang et al. 2020). The study used intensive atorvastatin treatment in combination with bone marrow-derived mononuclear cells rather than MSCs. The Phase 2 study was intended to "precondition" the cardiac microenvironment to make it less harsh and conducive for the transplanted cells to support their survival and reparability. A total of 100 patients (30–80 years) having LVEF <45% were enrolled in the study to receive either normal (20 mg/day) or high (80 mg/day) doses of atorvastatin with intracoronary mononuclear cells or placebo injections 2–4 weeks after STEMI. One year follow-up studies revealed that cell transplantation significantly improved LVEF as compared to the total placebo-treated patients. Only atorvastatin + mononuclear cell-treated patient group showed significantly improved LVEF as compared with placebo + atorvastatin-treated patients. Similarly, PET revealed significantly decreased fibrosis and increased viable myocardium in atorvastatin + mononuclear cell-transplanted animals. These data supported the combined atorvastatin and mononuclear cell-based therapy as more efficacious as compared to either of the treatment approach alone. The augmented efficacy of mononuclear cell-based therapy was attributed to the cytoprotective effects of atorvastatin on the infarcted myocardium, thus rendering it favorable for the transplanted cells.

Some earlier clinical studies have also reported significant mobilization of endothelial progenitor cells and homing in to the heart in response to atorvastatin treatment from the bone marrow and home-in to the myocardium after myocardial infarction (ClinicalTrials.gov Identifier: NCT00536887) (Hong et al. 2010) and coronary artery bypass surgery (ClinicalTrials.gov number, NCT01096875) (Baran et al. 2012).

Conclusion and Future Perspective

The modest outcome of most clinical trials in regenerative medicine has been attributed to the quality of the cell preparation besides the cytokine-rich harsh microenvironment of the infarcted myocardium, both of which interject to the massive death of the transplanted cells post engraftment. Hence, preconditioning of the donor cells to develop "super cells" (Haider & Ashraf, 2007) that could withstand the rigors of the infarcted heart and "fertilizing" the cardiac milieu (Xu et al. 2019; Yang et al. 2020) to make it favorable for the transplanted cells, especially during acute phase after infarction episode, remains the cornerstone of the ongoing research in cardiovascular regenerative medicine. In this regard, pharmacological preconditioning approach has come a long way in addressing both cell quality-related as well as cardiac milieu-related issues to achieve optimal outcome of cell-based therapy. The primary advantage of pharmacological preconditioning approach is that the drug molecules used in there already have an established safety profile for human use.

As discussed earlier, statins are known for their significant cytoprotective effects on MSCs and cardiomyocytes during in vitro and in vivo experimental animal models and during clinical studies in myocardial damage repair. Preconditioning of MSCs with statins before transplantation and combined treatment of cell therapy

with statin therapy has given encouraging results in the translational as well as clinical settings. The synergy between the intrinsic antiapoptotic and immunomodulatory properties of MSCs and pleiotropic action of statins, i.e., endothelial protection, immunomodulation, reduction in antioxidant stress, etc., together renders the cardiac environment favorable for the transplanted cells and augments their reparability. The combinatorial approach also involves stem cells from the intrinsic pool to home-in to the myocardium to participate in the myocardial repair and regenerative processes. Looking into the future, it is imperative that more research into the application of the different types of statins for myocardial damage repair be conducted. With the recent emergence of cell-free therapy approach, further characterization of statin-preconditioned MSCs-derived exosomes and their payload, and profiling of the conditioned medium from statin-preconditioned cells (Nowak et al. 2019), may offer ample opportunities for cardiovascular application to reduce morbidity and mortality due to myocardial injury. The potential of translating this research into successful clinical application looks bright; however, the statin-preconditioning regime should be tailored quite significantly depending on the nature and properties of the ischemic event. A summary of statin-based combined treatment approach with MSCs-based therapy for myocardial repair is outlined in Fig. 1.

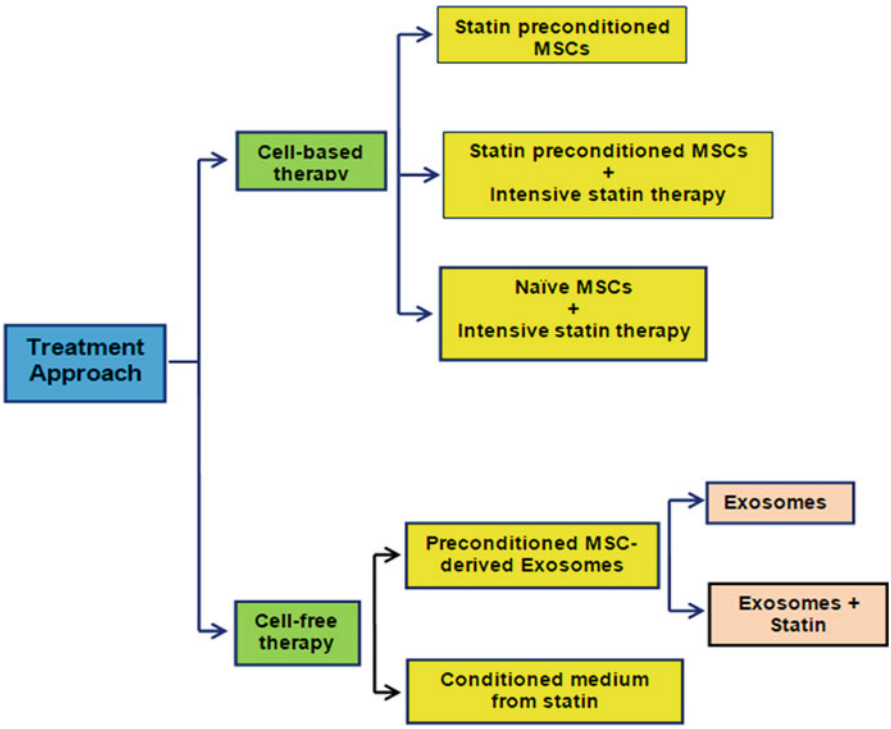

Fig. 1 Flow-diagram summarizing the cell-based and cell-free therapy approaches involving MSCs and statins

Cross-References

- ▶ Exosome-Based Cell-Free Therapy in Regenerative Medicine for Myocardial Repair
- ▶ Extracellular Vesicles-Based Cell-Free Therapy for Liver Regeneration
- ▶ Mesenchymal Stem Cells
- ▶ Mesenchymal Stem Cells for Cardiac Repair

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Unraveling the Mystery of Regenerative Medicine in the Treatment of Heart Failure

16

Mathieu Rheault-Henry, Ian White, and Rony Atoui

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M. Rheault-Henry · I. White · R. Atoui (✉)
Division of Cardiac Surgery, Health Sciences North, Northern Ontario School of Medicine, Sudbury, ON, Canada
e-mail: mrheaulthenry@nosm.ca; iwhite@nosm.ca

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Abstract

Despite many advancements that have been made in the field of cardiovascular research, heart failure is still associated with a poor prognosis and high mortality worldwide. The discovery that adult cardiomyocytes have an extremely low turnover rate has prompted researchers to investigate stem cells as a therapeutic option for cardiac regeneration. With stem cell therapy now approaching its third decade, numerous clinical trials have been completed and questions surrounding stem cell therapy are starting to be answered. It is now fairly well established that stem cell therapy has a positive safety profile, but only has produced neutral to moderately positive clinical outcomes. It is also clear that current stem cell therapies lack a significant ability to engraft and remuscularize the myocardium, suggesting cardiac repair occurs primarily through paracrine signaling mechanisms. Innovative strategies involving cardiac bioengineering, cell-free biomolecules, and combination therapies likely hold the key to advancing the field to the next stage. Looking ahead, we remain cautiously optimistic that stem cell therapeutics will have a significant place in the future of cardiovascular treatments, but there are still many questions that need to be answered before routine clinical application is possible.

Keywords

Cardiac · Clinical · Heart · Infarction · Regenerative medicine · Stem cells

Abbreviations

AAV	Adeno-associated virus
ACC/AHA	American College of Cardiology and the American Heart Association
ANP	Atrial natriuretic peptide
BMDSC	Bone marrow-derived stem cell
BMMNC	Bone marrow mononuclear cell
BNP	Brain natriuretic peptide
CABG	Coronary artery bypass grafting
CDC	Cardiosphere-derived cell
CDCP	Center for Disease Control and Prevention
CM	Cardiomyocyte
CPC	Cardiac progenitor cell
CSC	Cardiac stem cell
ECM	Extracellular matrix
EDV	End diastolic volume
EF	Ejection fraction
ESC	Embryonic stem cell
ESV	End systolic volume
FGF	Fibroblast growth factor
hESC-CP	Human embryonic stem cell cardiac progenitor
HF	Heart failure

HFpEF	HF with preserved ejection fraction
HFrEF	HF with reduced ejection fraction
IC	Intracoronary
IGF-1	Insulin-like growth factor-1
IM	Intramuscular
iPSC	Induced-pluripotent stem cell
IV	Intravenous
LV	Left ventricular
LVEDV	Left ventricular end diastolic volume
LVEF	Left ventricular ejection volume
LVESV	Left ventricular end systolic volume
MI	Myocardial infarction
MLHFQ	Minnesota Living with HF Questionnaire
MPC	Mesenchymal precursor cell
MSC	Mesenchymal stem cell
NYHA	New York Heart Association
PCI	Percutaneous coronary intervention
PDGF-B	Platelet-derived growth factor B
PSC	Pluripotent stem cell
SDF-1	Stromal cell-derived factor-1
SM	Skeletal myoblast
VEGF	Vascular endothelial growth factor

Introduction

Cardiovascular disease is a significant worldwide health issue that consistently ranks among the leading causes of death and disability-adjusted life years lost (Abbafati et al. 2020). Cardiovascular disease is an umbrella term used for numerous cardiac and peripheral vascular pathologies including heart failure. Heart failure (HF) is a global epidemic that impacts the quality of life, life expectancy, and the economics of the health care system. In the USA and Europe, HF is the leading cause of hospitalization with over one million admissions as a primary diagnosis (Ambrosy et al. 2014). Moreover, it represents up to 2% of total hospitalizations in these regions, with minor improvements in post-discharge mortality rates. It is estimated that there are over six million people in the USA living with HF, and this number is expected to increase to over eight million by 2030 (Mozaffarian et al. 2016; Virani et al. 2021). Globally, HF affects 26 million people, imposing an immense burden on international health care systems and society as a whole (Cheung and Jahan 2020).

Heart Failure

Heart failure is a complex clinical syndrome characterized by structural and functional abnormalities which impair ventricular filling or ejection of blood

(Gaglianello et al. 2016). These changes in cardiac function are highly problematic as they result in inadequate perfusion of vital organs and peripheral tissues. This mechanical pump failure consequently results in an inability of the heart to meet the body's metabolic demands. Heart failure is commonly classified as HF with reduced ejection fraction (HFrEF) or HF with preserved ejection fraction (HFpEF). HFrEF is defined as a left ventricular ejection fraction (LVEF) of less than 40%. Conversely, HFpEF is defined as an LVEF greater than 50% (Gaglianello et al. 2016). These classifications are essential, as they provide the information necessary to determine the appropriate management strategy and prognosis of the disease. Another important point to consider is the clinical severity of HF, which is graded based on the New York Heart Association (NYHA) functional classification. It defines four functional classes of heart failure – from class 1 being the least severe to class IV being the most severe (Inamdar and Inamdar 2016). Patients can move between classes relatively quickly as this classification system focuses on symptoms only. Moreover, the American College of Cardiology and the American Heart Association (ACC/AHA) HF staging system was created to complement the NYHA functional classification. With the ACC/AHA classification, there is no moving backward to prior stages. Once symptoms have developed, the patient is in stage C HF and will never again be classified in stage B. Together, the NYHA functional classification and the ACC/AHA clinical tools are useful in estimating the progression and future outcome of the disease.

The causes of heart failure are numerous – coronary artery disease, diabetes, hypertension, idiopathic cardiomyopathies, pressure overload, volume overload, cardiotoxic drugs, metabolic conditions, and inflammatory and hereditary conditions are common culprits. Nonetheless, the most common etiology of HF remains myocardial infarction (MI) (Tanai and Frantz 2015). Myocardial infarction results in ischemia, necrosis of cardiomyocytes (CMs), and fibrotic scar tissue formation (Mouton et al. 2018). The extent of CM damage depends on the duration of ischemia and the size of the zone of infarction. If the MI is not treated on time, severe structural and functional impairments ensue. Following an MI, compensatory mechanisms such as activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system work together to maintain adequate cardiac output and blood flow to vital organs (Gaglianello et al. 2016). Over time, these responses are often insufficient as they result in fluid retention and adverse remodeling of the chambers of the heart. Cardiac remodeling typically results in CM hypertrophy, resulting in decreased cardiac contractility. Following these changes, a progressive decline in cardiac function is observed until the heart ultimately fails to pump completely (Tables 1 and 2).

Table 1 New York Heart Association Functional Classification of Heart Failure

Class	Symptoms
I (Mild)	No symptoms[a] during normal physical activity (walking, stairs)
II (Mild)	Mild symptoms[a] and slight limitations during normal physical activity
III (Moderate)	Moderate symptoms[a] with significant limitations in physical activity. Comfortable only at rest
IV (Severe)	Severe symptoms[a] that markedly limits activity and are present even at rest

[a]Symptoms – fatigue, chest pain, palpitations, dyspnea, and syncope

Table 2 American College of Cardiology/American Heart Association Heart Failure Staging System

Stage A	Patients at high risk for heart failure who have not yet developed structural heart changes
Stage B	Patients with structural heart disease (i.e., reduced ejection fraction, left ventricular hypertrophy, and chamber enlargement) who have not yet developed symptoms of heart failure
Stage C	Patients who have structural heart disease and have developed symptoms of heart failure
Stage D	Patients with refractory heart failure requiring specialized intervention (transplantation and left ventricular assist device)

Due to the high quality of medical care and revascularization techniques such as coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI), survival rates following MI have increased (Skinner and Cooper 2011; Ambrosy et al. 2014). However, as patient survival increases, cases of HF are rapidly growing. Symptoms of HF are frequently homogenous, yet the progression of the disease and the pathological processes are diverse, warranting a holistic approach to treatment. It is recommended that patients incorporate both pharmacological and nonpharmacologic therapies to improve the functional capacity of the heart – reducing its workload (Gaglianello et al. 2016). Triple therapy, consisting of diuretics, angiotensin-converting enzyme inhibitors (ACE inhibitors), and beta-blockers, are some of the most common symptom management strategies for HF patients and have been particularly beneficial in HFpEF. Moreover, implantable cardiac defibrillators and ventricular assist devices are options for more severe cases. Heart transplantation is considered the gold standard for treatment, yet it yields a high cost, immune reactions are common, and there remains a major discrepancy between the availability of donors and recipients (Rojas and Haverich 2019; Mohite et al. 2015; Liu et al. 2020). Despite many therapeutic options available, the prognosis of HF remains poor, with a 5-year mortality rate of around 50% and a 10-year survival rate of only 20% (Gaglianello et al. 2016). These statistics warrant a treatment that can stop the progressive nature of the disease while restoring cardiac function. Due to the limited endogenous regeneration potential of the heart, stem cells as a therapeutic option to treat HF have gained interest globally. In the last few decades, stem cells have been extensively studied to treat patients living with HF.

Regenerative Potential of Stem Cells

The use of stem cells to generate healthy cells or replace diseased cells is not a novel idea. Historically, doctors have performed stem cell transplants – also known as bone marrow transplants. In these transplants, stem cells replace cells that have been damaged by disease or chemotherapy and serve as a vehicle for the recipient of these cells to fight off various forms of hematological cancers – leukemia, lymphoma, and

multiple myeloma. In regenerative medicine, the unique potential of stem cells is based on two important qualities: the ability to self-replicate and the potential to differentiate into mature, functional cell phenotypes (Zakrzewski et al. 2019).

Stem cells can be divided into two broad categories – pluripotent stem cells (PSCs) and adult stem cells. Adult stem cells are more differentiated and are limited to give rise to only certain sets of cells or tissues of the body (Singh et al. 2016). These can be broken down into derivatives from the bone marrow, cardiac tissue, skeletal muscle, umbilical cord, and adipose tissue. Human ESCs are termed pluripotent, meaning that they can divide into any cell type found in the body. These cells are incredibly versatile and can be used to repair or regenerate damaged or diseased tissues and organs. However, they are limited by important ethical implications as they arise from the blastocyst of an embryo. Novel protocols have been developed to reprogram adult stem cells to have properties of embryonic stem cells (Takahashi and Yamanaka 2006). The cells termed induced pluripotent stem cells (iPSCs) were developed by genetic reprogramming techniques to alter the potency level of mature somatic cells.

When considering stem cells as an option for cardiac regeneration, some cells are more specialized than others. Also, they can secrete different levels or combinations of various bioactive messengers, and some have different functions altogether through the expression of various cell markers (Sobhani et al. 2017). Stem cells can be transplanted directly into the heart, allowing engraftment, direct differentiation, and replacement of diseased cells. Conversely, some stem cells trigger a paracrine effect, involving the secretion of various chemical messengers that stimulate the patient's nearby cells to repair damaged tissue. To recognize and compare the therapeutic potential of the various stem cell types, it is important to understand their therapeutic potential along with the mechanisms in which they work. To date, ESCs, iPSCs, and various adult stem cell lineages such as bone-marrow-derived stem cells (BMDCs), cardiac stem cells (CSCs), and skeletal myoblasts (SMs) have either been tested or are currently undergoing clinical trials in the treatment of cardiac disease (Fig. 1).

Endogenous Cardiac Regeneration

It is well known that the human heart lacks any significant regenerative capacity in response to myocardial injury or chronic disease. This explains why myocardial injury is so problematic. An acute left ventricular MI that kills over 25% of the CMs in the LV can ultimately lead to chronic heart failure (Murry et al. 2006). Although the heart may not adequately repair itself after injury, it is becoming increasingly clear that the heart is not a postmitotic organ. Research in the last couple of decades has revealed that CM renewal does occur, albeit at a very slow rate. The current consensus is that in a healthy, adult human heart, the CM turnover rate is approximately 0.5–1% per year, and this rate decreases over the life span (Eschenhagen et al. 2017). A study using carbon 14 dating techniques in human cardiomyocyte DNA demonstrated that adult CM turnover takes place at a rate of 1% annually at age 20 and 0.3% at age 75. This

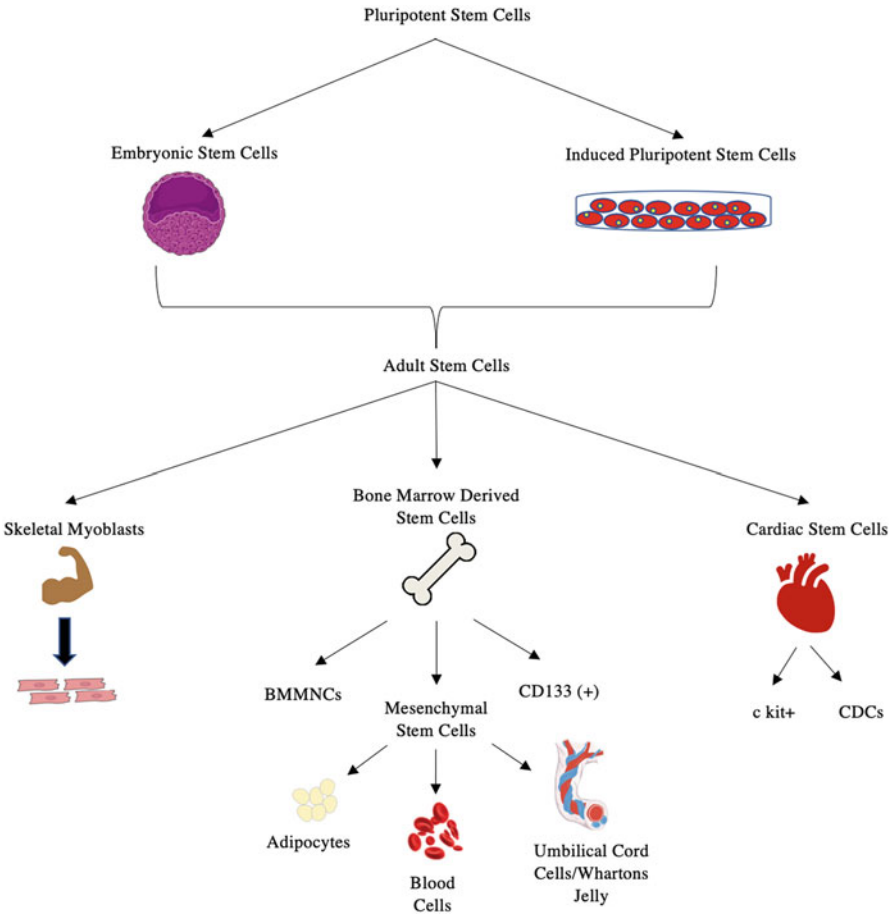

Fig. 1 Stem cell lineages utilized in regenerative medicine from least differentiated to most differentiated

equates to approximately 50% of the entire cell population being turned over in one's lifespan (Bergmann et al. 2009). This evidence was corroborated in a separate study that again demonstrated that CM renewal is greatest during the neonatal period and decreases in adulthood to less than 1% annually. Of note, turnover rates of cardiac endothelial cells and mesenchymal cells (MSCs) were slightly higher, at >15% and <4%, respectively (Bergmann et al. 2015).

Interestingly, anecdotal evidence supports the claim that cardiac regeneration in neonatal populations may be higher than initially thought. In 2015, a case report was published on a newborn child with thrombotic occlusion of the left anterior descending artery for >20 h, causing severe acute myocardial infarction. The myocardial damage was marked by elevated troponin levels, electrocardiogram, echocardiography, and cardiac angiography abnormalities. Miraculously, within

weeks the child's heart function began to recover, and at 1-year post-MI, there was no distinguishable difference in heart function or morphology compared to a healthy 1-year-old infant. A similar phenomenon of functional cardiac repair has been demonstrated in newborn murine models of severe MI and cardiac apex resection (Haubner et al. 2012; Porrello et al. 2011). This raises a pertinent question regarding the mechanism of new cardiomyocytes regeneration. Unfortunately, the answer remains elusive. Researchers hypothesize that new cardiomyocytes possibly originate from three potential sources: resident cardiac progenitor cells, proliferation of preexisting cardiomyocytes, or migration of extra-cardiac stem cells to the myocardium (Eschenhagen et al. 2017). We also do know that during embryonic development the heart enlarges primarily through CM proliferation. One month after birth, CM numbers are already at their highest and will remain constant across one's lifetime. In the second decade of life, cardiac growth occurs via cellular hypertrophy, and an increase of cardiomyocyte DNA content via polyploidization (Bergmann et al. 2015).

Determining the source and the extent of normal physiologic cardiac regeneration and renewal of CMs post-injury is a delicate but critical task. Identifying pathways that could be manipulated and amplified for therapeutic purposes remains a key goal for future research. Given that the endogenous turnover of CMs is relatively low, prevention of proapoptotic pathways immediately after cardiac injury and the stimulation of endogenous proliferative pathways may be the key to the cardiac regeneration puzzle. For example, researchers have recently begun investigating the Hippo-YAP pathway, with the hope of manipulating this intrinsic regenerative mechanism to influence cardiac regeneration.

Outcomes of Clinical Studies

Successful stem cell therapy depends on a multitude of outcomes that are observed in patients post-administration. Improvements in mortality, morbidity, and quality of life indexes such as the NYHA functional class, Minnesota Living with HF Questionnaire (MLHFQ), and distance on the six-min walk test with exercise capacity are commonly taken into consideration. In terms of heart function, stem cells should be able to improve left ventricular ejection fraction (LVEF), decrease end-systolic volume (ESV), end-diastolic volume (EDV), atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP). Lastly, the successful application of stem cells in HF relies heavily on the capacity for engraftment and survival of such cells into the host myocardium, the potential for revascularization and angiogenesis, and the capacity to electromechanically couple with resident CMs, allowing them to beat as a functional syncytium (Gerbin and Murry 2015). Most importantly, successful therapy needs to demonstrate a favorable safety profile, with minimal to no adverse events. Common concerns would be any cardiovascular events, cardiac arrhythmias, immune rejection, and teratoma formation. Past, present, and future clinical trials using various types of stem cells as a treatment for cardiac disease, and specifically HF, will be discussed in the following section in detail.

Pluripotent Stem Cells

In an attempt to regenerate the myocardium, some researchers have adopted an upstream approach to cellular-based therapies, using pluripotent stem cells (PSCs) and differentiating them into cardiac progenitors or functional CMs. The two basic approaches to creating PSC-derived CMs begin with either the extraction of human ESCs or the creation of iPSCs from mature somatic cell types. PSCs possess the unique ability of unlimited self-renewal and the ability to differentiate into functional progenitor cell types (Kadota and Shiba 2019). These two unique qualities allow researchers to differentiate a significant number of PSCs into functional CMs (Kadota and Shiba 2019). The newly generated CMs can then be implanted into the damaged tissue, with the ultimate goal of integration into the host myocardium. Recently, there has been increasing preclinical evidence accumulating on the efficacy of both iPSCs and human ESCs in the treatment of HF. Researchers have demonstrated that human PSCs can be successfully differentiated into functional CMs that display necessary electrophysiologic properties, calcium handling abilities, and contractile proteins (Kadota et al. 2013). Various animal models including rat, pig, and nonhuman primates have been used to show that human-ESC-derived-CMs can functionally engraft into the host myocardium and improve contractile function (Kadota et al. 2020).

To date, the phase I ESCORT trial (NCT02057900) conducted out of France is the only published clinical trial investigating human PSC-CMs in humans with HF (Menasché et al. 2018). This trial investigated the safety and feasibility of implanting human-ESC-derived CD15+ Isl-1+ cardiac progenitor cells (hESC-CP) in patients with severe ischemic left ventricular dysfunction. Six patients had a fibrin patch embedded with hESC-CPs transplanted epicardially as an adjunct to CABG. Safety was measured at 1-year follow-up for three primary endpoints: cardiac or off-target tumors assessed by CT and PET scan imaging, arrhythmias detected by serial interrogations of cardioverter-defibrillators, and alloimmunization assessed by detection of donor-specific antibodies. During 1-year follow-up, none of the patients exhibited any arrhythmias, and no tumors were detected in any of the patients included in the follow-up. Three patients developed low-level donor-specific antibodies; however, none of the patients had any clinically relevant complications due to the alloimmunization. Although efficacy was not a primary outcome in the study, all patients at the 1-year endpoint demonstrated symptomatic improvement from the baseline determined by a decrease in the NYHA functional class score and improvements in distance during the 6-min walk test. Patients also showed a modest increase in LVEF and a significantly improved left ventricular wall motion score measured via transthoracic echocardiogram. Although there is only one completed clinical trial using PSC-CMs in humans, there are currently two ongoing clinical trials utilizing iPSCs-CMs and likely several others in the near future. The first trial is an open-label clinical trial in China (HEAL-CHF, NCT03763136) assessing the safety and feasibility of direct epicardial injection of allogeneic human iPSCs-CMs in five patients with HF. The second clinical trial was launched in Japan in 2020 to implant cellular sheets of iPSCs-CMs into patients with ischemic cardiomyopathy. There are

relatively few known details about the trial, but it is enrolling ten patients over 3 years and aims to assess the efficacy and safety of iPSC-CMs in humans at the 1-year postoperative stage (Cyranoski 2018).

There remain some real concerns regarding the use of iPSC-CMs in clinical settings. Preclinical trials in porcine and nonhuman primate models demonstrated nonlethal transient ventricular arrhythmias posttransplantation assessed by electroanatomical mapping (Shiba et al. 2016; Romagnuolo et al. 2019). Incidentally, the arrhythmias were shown to be of graft-site origin. Two proposed mechanisms are believed to be contributing to the ectopic pacing: immaturity of the neomyocytes and automaticity (Kadota et al. 2020). No arrhythmias were observed in the human clinical trial. Besides arrhythmias, poor long-term engraftment of PSC-CMs remains a primary concern and this issue has been discussed in depth in a later section of the chapter. Lastly, immune rejection of the allogeneic donor cells is a potential problem that necessitates immunosuppression. However, Menasché et al. (2018) have demonstrated that three patients developed alloimmunization, but it was clinically silent.

Skeletal Myoblasts

Skeletal myoblasts (SMs) are harvested under the basal lamina of muscle fibers. This cell lineage is an attractive option for regenerative therapy as it provides an abundant source of readily available cells with myogenic differentiation potential (Haider et al. 2004a). Moreover, they can be easily expanded undifferentiated in culture to achieve the required number for cell-based therapy (Sim et al. 2003). Interestingly, SMs are immuno-privileged and need only transient immunosuppression for successful cross-species transplantation (Haider et al. 2004b). These data have led to first-in-man allogenic SMs transplantation in patients (Law et al. 2003). Preclinical trials found that SMs could improve cardiac function by differentiating into functional CMs in animal models (Taylor et al. 1998; Chiu et al. 1995; Ye et al. 2007). SMs are excellent carriers of therapeutic genes due to their robust nature. Hence, they have been genetically modulated for the angiomyogenic repair of the heart in experimental settings (Lei et al. 2007, 2011). They have also been preconditioned to enhance their post-engraftment survival and improve their reparability (Niagara et al. 2007; Elmadbouh et al. 2011). SMs have also been reprogrammed to develop induced pluripotent stem cells for myocardial repair, however, not without the possibility of myocardial tumorigenesis (Ahmed et al. 2011a, b).

Researchers quickly pushed SMs into human clinical trials with hopes to achieve similar results (Menasché et al. 2001; Sim et al. 2003; Siminiak et al. 2004). Unfortunately, researchers could not reproduce the successful findings in human clinical trials. The MARVEL study tested the intramyocardial (IM) injection of SMs in a sample of 14 patients, but ultimately resulted in an increased risk of developing ventricular tachycardia (Povsic et al. 2011). In addition, there were no significant improvements in LVEF or Minnesota Living with HF scores at the 6-month follow-up. The SEISMIC trial investigated the IM transplantation of autologous SMs in a cohort of 40 patients. It also found that LVEF and global heart function

improvements were not statistically significant at 6 months following administration (Duckers et al. 2011). Serious adverse events were common, yet there were no differences in incidence between the cell therapy group and the control group.

Similar to the MARVEL and SEISMIC studies, the MAGIC trial also administered SMs via IM route. In a sample of 120 patients, it was found that SMs did not improve LVEF or global cardiac function (Menasché et al. 2008). This was confirmed on a 5-year follow-up (Brickwedel et al. 2014). More significant risks of arrhythmias were noted upon administration of SMs and this was determined to be the primary concern regarding this cell lineage. Skeletal myoblasts are deprived of gap junctions and therefore lack electromechanical coupling potential, forming the basis of arrhythmia formation (Reinecke et al. 2002; Abraham et al. 2005). Though more studies with larger sample sizes could indicate whether or not SMs could be incorporated in HF treatment in the future, researchers have transitioned away from this phenotype due to their apparent lack of efficacy and safety concerns.

Bone Marrow-Derived Stem Cells

Bone marrow-derived stem cells are one of the most heavily tested cells in the treatment of cardiac disease to date. Researchers believe that autologous bone marrow mononuclear cells (BMMNCs) can improve heart function through angiogenesis and direct cardiomyocyte regeneration within the myocardium (Hu et al. 2011). The first ever published clinical trial using autologous (BMMNCs) included 21 patients with chronic HF who received the cells via transendocardial route. There were no safety concerns, and after 4 months, significant increases in LVEF, reductions in ESV, and improvements in perfusion and myocardial contractility were observed (Perin et al. 2003). Other clinical trials, such as the TOPCARE-CHD, showed significant improvements in global cardiac function, regional contractility, and mortality besides a decrease in ANP and BNP in response to transcoronary BMMNCs injection (Assmus et al. 2007). Similarly, improvements in LVEF, infarct zone, exercise capacity, NYHA functional class, and long-term mortality were observed up to 5 years after intracoronary (IC) administration of autologous bone marrow cells in the STAR-heart study (Strauer et al. 2010). The initial success of bone marrow-derived stem cells (BMDSCs) prompted the creation of more extensive phase II trials such as the FOCUS-CCTRN and the CELLWAVE, where autologous BMMNCs were administered via transendocardial and IC route, respectively. The initial excitement created by the smaller clinical trials data was short lived, as no significant improvements in LVEF, maximal oxygen consumption, infarct size, and reversibility of ischemia were observed in the larger phase II trials (Perin et al. 2012; Assmus et al. 2013). In the TAC-HFT trial, patients received either transendocardial injections of autologous BMMNCs, autologous MSCs, or placebo. Results showed that only MSC-based therapy decreased infarct size and improved the 6-min walk test distance and regional function of the heart (Heldman et al. 2014). No improvement was noted in LVEF. The Cardio133 clinical trial reported significant adverse events in patients receiving CD133(+) bone marrow cells delivered via

CABG. Although some improvements in scar size and perfusion were observed, it was found that the injection of CD133(+) cells had neither effect on clinical symptoms of HF nor global LV function (Nasseri et al. 2014).

A systematic review and meta-analysis including 1907 participants and a total of 38 randomized controlled trials found low-quality evidence that BMDSCs improved LVEF and mortality during short-term and long-term follow-up (Fisher et al. 2016a). Moreover, there was little evidence that BMDSCs improved NYHA functional class in HF patients. Though periprocedural adverse events were uncommon and serious adverse events were rare, there is no current consensus on whether or not this cell type is truly efficacious in improving outcomes. Fisher et al. applied the trial sequential analysis of two Cochrane reviews to overcome the limitations of meta-analyses (Fisher et al. 2016b). Randomized controlled trials using autologous BMDSCs to 2739 patients with acute MI and 1094 patients with HF were included in the analysis. It was found that although there is insufficient evidence to determine the treatment effect in acute MI, there is solid evidence that the administration of autologous BMDSCs reduced mortality and rehospitalization for those with HF. It was also found that BMDSCs did not improve LVEF by more than a mean difference of 4% in patients with acute MI or HF (Fisher et al. 2016b). Results of this analysis must be confirmed by an adequately powered double-blind phase III trial. Given these marginally positive findings, there is currently no consensus on whether or not BMDSCs will have a future role in treating HF patients. However, BMDSCs seem to have a favorable safety profile as there are generally few safety concerns.

Mesenchymal Stem Cells

Mesenchymal stem cells are a subtype of BMDSCs. Though found primarily in the bone marrow, they are also located in other areas of the body, including adipose tissue, blood, and the umbilical cord, commonly referred to as Wharton's jelly (Menasché 2018; Mathiasen et al. 2020; Zhao et al. 2015). Among the different BMDSCs types, MSCs primarily act via paracrine signaling mechanisms and, for this reason, seem to show great promise for regeneration of the myocardium (Menasché 2018). Paracrine signaling allows for pro-angiogenic effects by releasing vascular endothelial growth factor (VEGF), insulin growth factor-1 (IGF-1), and stem cell growth factor (Natsumeda et al. 2017; Liu et al. 2020). Moreover, the paracrine effect stimulates the proliferation and differentiation of endogenous cardiac progenitor cells (CPCs) (Natsumeda et al. 2017). These CPCs have pro-angiogenic capabilities and the potential to promote the differentiation of existing cardiac cells, though evidence of their long-term engraftment is lacking (Williams et al. 2013). In addition to paracrine signaling, MSCs can directly contribute to vascular proliferation and direct myocardial regeneration on a greater scale (Tehzeeb et al. 2019; Lalu et al. 2018). This cell lineage also has important repair properties through immunomodulation, anti-fibrotic, pro-angiogenic, and anti-oxidative effects (Turner et al. 2020). One unique characteristic of MSCs is

that they do not express major histocompatibility complex class II (MHC II) antigens. Hence, MSCs are good candidates for allogeneic sourcing, as they are nonimmunogenic (Nair and Gongora 2020). Together, these qualities make MSCs great contenders for treating chronic HF. A meta-analysis investigating 52 preclinical studies showed that MSCs were moderately associated with significant improvements in LVEF and showed that cardiac cell therapy is safe and not associated with increased mortality (van der Spoel et al. 2011). A recent systematic review investigated the impact of MSCs as a treatment for nonischemic dilated cardiomyopathy (Hoeeg et al. 2020). In total, 27 studies were included; however, most studies involved preclinical animal models (3 clinical and 24 preclinical trials). Of these, 21 of the included trials tested bone marrow-derived MSCs, 4 tested human umbilical cord MSCs, and 3 tested adipose tissue-derived MSCs. It was found that bone marrow, umbilical cord, and adipose tissue-derived MSC treatment can improve cardiac function in nonischemic dilated cardiomyopathy through main mechanisms of anti-fibrotic and anti-apoptotic effects, angiogenesis, and immunomodulation. This suggests that independent of the cell origin, all MSC types share synonymous mechanisms of action. All three clinical trials and 22 of the 24 preclinical trials reported improvements in cardiac function following administration of MSCs.

Thus far, human clinical trials utilizing MSCs in HF have yielded exciting results. The results of the first placebo-controlled trial, MSC-HF, indicated that the IM injection of autologous MSCs is safe, reduces hospital admissions, and improves myocardial function (Nigro et al. 2018). In addition, the TAC-HFT trial suggested that MSCs were superior to BMMNCs in reducing infarct size and improving regional heart function in patients with chronic HF (Heldman et al. 2014). Other studies like the POSEIDON trial compared the transendocardial delivery of autologous versus allogeneic MSCs in patients with HF. Interestingly, both autologous and allogeneic MSCs improved LV function while reducing infarct size and adverse cardiac remodeling without any safety concerns highlighted (Hare et al. 2012). Five years later, results of the POSEIDON-DCM trial were released. Overall, it was demonstrated that more significant improvements were observed in allogeneic MSCs versus autologous MSCs in patients with nonischemic dilated cardiomyopathy. There were more significant improvements in quality of life, functional capacity scores such as the NYHA functional class and MLHFQ, the 6-min walk test, and improvements in ejection fraction (Hare et al. 2017). Just like the POSEIDON trial, the transendocardial delivery of both autologous and allogeneic MSCs in the POSEIDON-DCM trial had no notable safety concerns. Though more extensive trials are required to determine which cell source is more favorable, evidence supports the superiority of allogeneic MSCs regarding the efficacy and endothelial function. Another trial led by Mathiasen et al. (2020) administering MSCs via IM injection resulted in improvements in LVEF, stroke volume, and myocardial mass in patients with HF. A larger trial, the DREAM-HF trial with 566 patients enrolled, was recently completed. It evaluated the efficacy of allogeneic mesenchymal precursor cells (MPCs) in patients with advanced chronic HF. Results are pending.

A systematic review and meta-analysis investigating the efficacy and safety of MSCs in ischemic and nonischemic cardiomyopathies determined that of the 29 clinical trials, the vast majority demonstrated improvements in LVEF, LVESV, NYHA functional class, and exercise capacity without significant safety concerns (Poulin et al. 2016). Patients who received stem cells as an adjunct to CABG had the most remarkable improvements in LVEF, which justifies the role of catheter-based revascularization. Based on this data, it seems that MSC therapy may be a feasible option in improving cardiac function while decreasing adverse cardiac remodeling in patients with HF. Another systematic review and meta-analysis reviewed the safety and efficacy of MSCs in 12 trials focused on ischemic HF. There were significant improvements in LVEF; however, there were no significant improvements in mortality. Additionally, several studies within the meta-analysis showed an increase in quality of life and physical performance. However, quality of life and performance status were inconsistently reported in studies, which limited the ability to provide conclusions (Lalu et al. 2018). Most importantly, there seems to be no association between treatment with MSCs and adverse events, suggesting a favorable safety profile.

Other descendants of MSCs such as cardiopoietic and umbilical cord MSCs have been studied in clinical trials. These are more specialized cells derived from a pure MSC lineage. Cardiopoietic stem cells are derived from MSCs in the bone marrow and are of particular interest in regenerative medicine. One of the first using cardiopoietic cells, the C-CURE trial, demonstrated significant improvements in LVEF, quality of life, and lower LVESV 2 years after administration into the heart with no safety concerns noted (Bartunek et al. 2013). This small-scale study catalyzed the formation of the CHART-1 trial, which had a greater sample size of 351 individuals. Interestingly, this study shared similar results to the C-CURE trial, indicating that cardiopoietic cells have the potential to provide long-lasting benefits toward cardiac function in those with HF (Teerlink et al. 2017; Bartunek et al. 2017). Wharton's jelly, a gelatinous substance present within the umbilical cord, is rich in MSCs, which have been tested in various clinical trials. Of these, a study led by Zhao et al. (2015) studied the delivery of umbilical cord MSCs via IC route in combination with various medications, such as ACE inhibitors or ARBs, beta-blockers, diuretics, and digoxin. Twenty-four hours after transplantation, symptoms of HF such as cough, dyspnea, and chest tightness were alleviated, though no improvements were noted in LVEF. There were some improvements in the 6-min walk test, NT-pro BNP levels were significantly lower than the control group, and improvements in mortality rates were observed. Given these positive results, it is crucial to link these improvements with the medications given in combination with the MSCs. In the RIMECARD trial, the intravenous (IV) infusion of umbilical cord MSCs were found to improve LVEF but did not reduce LVESV or LVEDV (Bartolucci et al. 2017). No significant safety concerns were noted.

Though MSCs have massive potential in regenerative medicine, there is a need for larger, international clinical trials to fully elucidate the field of MSC therapy in humans living with HF. The surge of incoming clinical trials, including the first phase III clinical trial, should help clarify the true therapeutic potential of MSCs in HF. Put together, the meta-analysis by Lalu et al. demonstrated that MSCs could

improve LVEF and enhance the quality of life and performance, that too without any major safety concerns (Lalu et al. 2018).

Cardiac Stem Cells

Cardiac stem cells (CSCs) were among the most heavily researched cells in cardiac regenerative medicine. Clinical research led by Dr. Piero Anversa showed great promise for the application of CSCs to treat heart failure. He claimed that CSCs produced functional myocardial changes and they were a viable option to treat heart failure. These claims sparked a great interest in the medical community and the public (Chien et al. 2019). Many researchers attempted to replicate Anversa's studies albeit without success. It was soon discovered that the field of CSCs was heavily compromised and Anversa and his group were accused of scientific misconduct. As a result, the Brigham and Women's Hospital along with the Harvard Medical School took action by launching an investigation on Anversa's work. In 2014, the SCIPIO clinical trial using c kit+ CSCs was retracted, and by 2018, the investigations revealed that 31 publications contained falsified or fabricated data (Ozkan 2019). The National Institute of Health also stepped in by suspending the CONCERT-HF trial due to its lack of scientific foundations (Bolli et al. 2018). This clinical trial was the first to evaluate the combination of CSCs and MSCs as a treatment of HF.

To date, c-kit+ CSCs and cardiosphere-derived cell (CDC) phenotypes have been studied in clinical trials. The CADUCEUS trial demonstrated that the IC injection of CDCs reduced scar tissue size and improve regional contractility and heart mass on MRI (Makkar et al. 2012). There were no differences in ESV, EDV, and LVEF. There were no significant adverse events, alluding to a favorable safety profile for CDCs. The TAC-HFT-II trial will soon compare therapy with autologous MSCs alone vs MSCs combined with c-kit+ CSCs (Bolli et al. 2018). Without question, the implications of Piero Anversa's 31 retracted studies will have a long-lasting effect on the field of CSCs. The findings of these investigations have created a significant distrust of the scientific community and discredited the current advancements made in this field. Although other clinical trials are currently investigating the feasibility and efficacy of CSCs and cardiac-derived stem cells, clinical benefit has yet to be demonstrated for patients. Moreover, CSC isolation is invasive as it requires a heart biopsy, and culture requires many days before injecting adequate numbers (Nigro et al. 2018). In the future, it is of utmost importance that rigorous scientific standards are followed when conducting clinical trials to protect the integrity of research and protect patients (Tables 3 and 4).

Understanding the Factors Affecting Cell-Based Therapy

Though the safety profile of stem cells appears to be satisfactory, their overall efficacy is, at best, modest. For successful cell therapy to treat HF, a greater understanding of the factors surrounding the application of cellular treatment is warranted.

Table 3 Summary of safety parameters in human clinical trials

Cell type	Safety parameters in human subjects	References
ESCs	• Positive safety profile in one human clinical trial • Silent alloimmunization in three of six patients • Larger trials are warranted	Menasché et al. (2018)
iPSCs	No published human clinical trials completed	Cyranoski (2018)
Skeletal myoblasts	• Risk of ventricular arrhythmias • Easy to harvest	Menasché et al. (2008), Povsic et al. (2011), and Brickwedel et al. (2014)
BMDSCs	• Positive safety profile demonstrated in allogeneic and autologous human clinical trials • Easy to harvest • Decrease in arrhythmogenic risk • Noteworthy increase in adverse events	Hu et al. (2011), Perin et al. (2003), Strauer et al. (2010), Assmus et al. (2013), and Hu et al. (2011) Strauer et al. (2010) Nasseri et al. (2014)
MSCs	• Positive safety profile demonstrated in multiple human clinical trials • Dyspnea, fatigue, and chest tightness 1-month post-transplantation, though small sample	Mathiasen et al. (2020), Hare et al. (2012, 2017), Bartunek et al. (2013), Bartolucci et al. (2017), Poulin et al. (2016), Lalu et al. (2018), and Zhao et al. (2015)
CSCs	• Heavily compromised field of research due to lack of scientific integrity • Isolation of cells is invasive • Suggested positive safety profile of CDCs	Ozkan (2019) Nigro et al. (2018) Makkar et al. (2012)

Engraftment, Survival, and Rejection

One of the most critical impediments in stem cell therapy is their ability to engraft and survive in the heart post-administration, while avoiding immune rejection from the recipient. Both preclinical and clinical trials show that cell retention in the heart 24 h post-administration does not exceed 10% (Hou et al. 2005; Aicher et al. 2003; Blocklet et al. 2006). This is likely due to a poor engraftment potential along with the rapid washout of cells once they are injected into the heart (Terrovitis et al. 2009). These shortcomings have prompted the development of improved cell retention approaches, such as plugging the injection site with a fibrin compound to prevent backflow of cells, and transplantation of constructed cell sheets besides the use of bioengineered natural or synthetic polymers (Chiu et al. 2012; Terrovitis et al. 2009). Autoimmune rejection of transplanted cells is another crucial risk to mitigate, mainly when the source is allogeneic. This has resulted in a push for cells that require minimal to no immunosuppression, such as ESC-derived cells, MSCs, and MPCs. Future research focusing on cell retention while decreasing the risk of immune rejection will continue to improve the efficacy and feasibility of stem cell therapy for HF.

Table 4 Landmark human clinical trials

Cell type	Clinical trial	Sample size	Results	References
ESCs	ESCORT	6	Improvement in NYHA functional class, 6-min walk test and heart wall motion	Menasché et al. (2018)
iPSCs	Cyranoski (2018)	10	Trial results pending	Cyranoski (2018)
	HEAL-CHF	2	Trial results pending	
Skeletal myoblasts	MAGIC MARVEL SEISMIC	120 23 40	No improvements in LVEF or global heart function No improvements in LV function Moderate improvements in 6-min walk test No improvements in LVEF at 6 months	Menasché et al. (2008) and Brickwedel et al. (2014) Povsic et al. (2011) Duckers et al. (2011)
BMMNCs BMMNCs and autologous MSCs CD133(+) BMDSCs	Perin et al. (2003) TOPCARE-CHD STAR-heart FOCUS-CCTRN Hu et al. (2011) TAC-HFT CARDIO33 Systematic review and meta-analysis Systematic review and meta-analysis	21 121 191 153 60 65 60 1907 1094	Improvements in LVEF, perfusion, and contractility Reductions in ESV Improvements in global cardiac function, contractility, and mortality Decreased ANP and BNP Improvements in LVEF, NYHA functional class, and long-term mortality Decreased LV preload, ESV, and infarct area No improvements in LVEF, maximal O_2 consumption, and infarct size Improvements in LVEF, LVESV, 6-min walk test, and exercise tolerance. Decreased BNP levels Decreased infarct size and improvements in 6-min walk test and regional function of the heart in MSC group only. MSCs better at improving myocardial function No improvements in LV function or clinical symptoms Improvements in mortality, LVEF, and NYHA functional class (low-quality evidence) Reductions in mortality and rehospitalization minimal improvements in LVEF	Perin et al. (2003) Assmus et al. (2007) Strauer et al. (2010) Perin et al. (2012) Hu et al. (2011) Heldman et al. (2014) Nasseri et al. (2014) Fisher et al. (2016a) Fisher et al. (2016b)
Autologous MSCs Autologous	MSC-HF POSEIDON POSEIDON-	60 31 37	Improvements in LVEF, stroke volume, and myocardial mass Improvements in LV functions with	Mathiasen et al. (2020) Hare et al. (2012)

(continued)

Table 4 (continued)

Cell type	Clinical trial	Sample size	Results	References
and allogeneic MSCs	DCM	36	reductions in adverse remodeling and infarct size	Hare et al. (2017)
Cardiopoietic	C-CURE	351		Bartunek et al. (2013)
Umbilical MSCs	CHART-1	30	Greater improvements seen in allogeneic MSCs regarding functional capacity, quality of life, EF, 6-min walk test, and the MLHFQ	Teerlink et al. (2017) and Bartunek et al. (2017)
Umbilical MSCs in collagen hydrogel	RIMECARD	59	Improvements in LVEF, quality of life, and decreased LVESV	Bartolucci et al. (2017)
Allogeneic MPCs	Zhao et al. (2015)	50	Decreased LVESV and LVEDV but no improvements in LVEF	He et al. (2020)
MSCs	He et al. (2020)	566	Improvements in LVEF but no improvements in LVESV or LVEDV	Borow et al. (2019)
	DREAM-HF	29 studies	No improvements in LVEF but some improvements in the 6-min walk test, mortality rate, and NT-pro BNP levels	Poulin et al. (2016)
	Systematic review and meta-analysis	12 studies	Improvements in LVEF, NYHA functional class, viable heart mass, and quality of life. Decreased LVESV and LVEDV	Lalu et al. (2018)
	Systematic review and meta-analysis		Trial results pending	
			Improvements in LVEF, LVESV, NYHA functional class, quality of life, and exercise capacity	
			Improvements in LVEF. No improvements in mortality or quality of life	
CSCs	CADUCEUS	31	Improvements in scar size, regional contractility, and heart mass; no differences in EDV, ESV, and LVEF between groups	Makkar et al. (2012)

Dosage

The optimal dosage of stem cells to reach therapeutic effect is still unknown, and the evidence is conflicting. Studies show large variations in dosing from 1×10^6 to 2×10^8 cells per dose administered per patient (Madonna et al. 2014). Though these include a large number of cells administered to a patient, it is not enough, as on average, one billion CMs are lost following an MI (Robey et al. 2008). There is a need for larger doses of stem cells to be administered if the goal is to replace lost CMs following MI. However, this may not be the case, as several clinical trials have demonstrated that smaller doses of stem cells are more effective than higher doses, possibly due to increased paracrine and cytoprotective factors release, activation of angiogenesis, and induced cardiomyocyte hypertrophy (Nigro et al. 2018). Whereas

large doses of stem cells may increase the potential for remuscularization, cell engraftment and survival are pretty low, and large doses may aggregate in areas of the heart, thus increasing the risk of arrhythmias (Prockop and Olson 2007). Also, the exact timing of cell administration has not been confirmed – though it is hypothesized that the longer the time interval between MI and administration of stem cells, the less the patient is likely to benefit. Current and future clinical trials will hopefully address these controversies and challenges.

Cell Type

Many different cell types have been tested in clinical trials, yet there is no current consensus on the type that is best suited for cardiac regeneration. In an ideal world, the perfect stem cell would be able to proliferate, engraft, and survive in ischemic areas, along with the ability to induce paracrine effects to stimulate endogenous cardiac regeneration. Moreover, it would have the potential to contract and electromechanically couple with the host CMs. At the moment, no cell has met all of these expectations in clinical trials (Gerbin and Murry 2015). The quality of the cell in question is also important to consider, as older age and preexisting comorbidities limit cell potency and regenerative potential (Nigro et al. 2018). Based on these findings, allogeneic sources of cells may present greater advantages over autologous sources. It is noteworthy that allogeneic cells can be cultivated and isolated from younger, healthy donors devoid of comorbidities and genetic defects. Allogeneic cells can be screened for quality and stored as an "off the shelf" product, making them readily available for acute applications. Lastly, allogeneic stem cells are more appealing in the eyes of the pharmaceutical industry as they provide superior profit margins versus autologous stem cells (Nigro et al. 2018). However, one must be wary of this conflict of interest, as we cannot sacrifice the quality of the product over the financial interests of external parties.

Currently, MSCs have been of particular interest in trials due to their ease of isolation and extraction, their multipotent differentiation potential, low immunogenicity, and their potential to trigger paracrine effects (Menasché et al. 2018; Nigro et al. 2018). Recent research has focused on more pure forms of stem cells, such as CSCs to increase the regenerative potential. Though CSCs have been shown to improve some aspects of cardiac function in patients with HF, the isolation of autologous CSCs is an invasive procedure, culture takes time, and the field of CSCs is heavily compromised (Nigro et al. 2018; Ozkan 2019). Some derivatives of iPSCs, such as cardiomyocyte-derived from human iPSCs, pose some risk of tumorigenicity (Nigro et al. 2018). For the moment, the most promising types of stem cells for cardiac regeneration appear to be iPSCs and ESCs. Alternatively, different approaches to cellular therapy may involve combining different cell types to increase efficacy. The rationale behind combining cells revolves around the activation of various regenerative pathways due to the underlying physiologic role of each cell type. Preclinical trials have shown promising results. It was found that the combination of MSCs and CPCs in the treatment of chronic HF improved cardiac

outcomes versus MSCs alone or placebo (Williams et al. 2013; Karantalis et al. 2015; Natsumeda et al. 2017). Though there were some differences between the three trials, there were improvements in EF, contractility, cell retention, and decreases in infarct size versus MSCs administered alone. Since MSCs alone act primarily via paracrine signaling, they stimulate angiogenesis and the proliferation, differentiation, and migration of endogenous CPCs to the heart (Natsumeda et al. 2017). However, the evidence on long-term engraftment is lacking (Williams et al. 2013). Other preclinical studies investigated the utility of epicardial patches containing human iPSC-derived CMs combined with human MSCs versus administering either of the cell type alone. It was found that the combination therapy group showed more significant improvements in EF, cardiac fibrosis, and capillary density (Park et al. 2019). Larger randomized, double-blind trials with more extended follow-up periods are warranted to determine which combination of cell types will yield the remarkable improvements and reduce safety concerns in HF patients. Alternatively, cardiac regeneration may not rely on stem cells after all. Current studies are now investigating the use of cell-free strategies due to concerns that the administration of stem cells results in poor cellular retention rate. This approach is discussed in greater detail in section "Cell-Free Strategies."

Route of Administration

An effective route of delivery is as important as the type of cell in question. It is one of the essential factors in successful stem cell treatment, as it can affect the potency of the cells, their retention, engraftment, and survival in the recipient's heart (Turner et al. 2020; Nigro et al. 2018). Successful delivery of cells will depend on the ability of the cells to migrate to the target area of the heart, their engraftment potential, and the ability to function in synchrony with the heart's natural rhythm without interference. Overall, an optimal delivery method should ensure the survival of the cells. It must be well tolerated by the patient, and the procedure should be relatively easy to perform by a clinician.

Preclinical studies have utilized various methods of delivery such as transendocardial, retrograde intracoronary sinus, open surgical epicardial injection, IM, IC, IV, and, more recently, 3D scaffolding with the help of cardiac patches (Bruyneel et al. 2016; Nakamura and Murry 2019; Mazzola and Di Pasquale 2020). The most common routes of administration include, IM, IC, IV, transendocardial, and 3D scaffolding.

Intramyocardial injection entails a direct injection of stem cells in a targeted area of the heart. These cells are usually injected along the borders of infarcted heart tissue as this provides better blood and oxygen supply for the cells to survive. This method ensures adequate blood supply to the cells, an essential component for their survival (Campbell and Suzuki 2012). This method of delivery provides the highest rate of retention, greatest engraftment, and remuscularization potential but fails to produce a significant paracrine effect (Nakamura and Murry 2019;

Campbell and Suzuki 2012). This technique is more invasive and risks myocardial perforation, vascular injury, arrhythmia, and embolism. Moreover, it can be challenging to distinguish between infarcted tissue and normal myocardium. For this reason, a skilled clinician is required.

The intracoronary infusion of stem cells is the most common and safer delivery method in clinical trials. This is primarily due to the central role of catheter-based revascularization of the heart in MI (Nakamura and Murry 2019; Tehzeeb et al. 2019). Clinical trials have deemed this method superior to the IM route as it promotes the paracrine effect, is less invasive, and ensures high cell survival rates due to the rich oxygen and nutrient content in the coronary circulation. The IC injection of cells is considered to decrease the risk of inflammation and damage to the myocardium post-transplantation, while allowing for a uniform distribution of cells in the target area (Campbell and Suzuki 2012). Given these slight advantages, IC injection is associated with long-term minimal cell retention due to rapid washout in humans, resulting in inefficient remuscularization of the heart (Nakamura and Murry 2019). Moreover, large doses of cells cannot be delivered via the IC route due to risk of obstruction of the coronary arteries, ultimately resulting in ischemia and myocardial cell death (Freyman et al. 2006; Goussetis et al. 2006; Nakamura and Murry 2019).

Although the IV approach has demonstrated positive safety parameters and is one of the least invasive methods, it is less efficacious than the IM and IC route (Freyman et al. 2006; Menasché 2018). This lack of efficacy is primarily due to a lack of cell retention, and engraftment and the majority of the cells remaining trapped in the lungs. Most of the cells are eliminated by phagocytic cells in the reticuloendothelial system (Freyman et al. 2006; Bruyneel et al. 2016; Turner et al. 2020). Moreover, there are concerns about vascular occlusion that can quickly occur with systemic delivery. To improve the effectiveness of the IV method, the approaches that will enhance the cellular homing mechanisms to the heart are essential.

The transendocardial route is the most challenging technique to execute, yet it can avoid the need for open-heart surgery – reducing periprocedural risks. It has shown tremendous potential for cell retention as it deposits stem cells directly into the myocardium (Tehzeeb et al. 2019; Turner et al. 2020). Like the IM route, there is a risk for ventricular rupture and arrhythmia formation. Though some small preclinical and clinical trials have shown the safety and efficacy of the transendocardial route, larger, more robust clinical trials are necessary to evaluate the long-term success of this method.

Bioengineering of cellular materials has recently entered the field of regenerative medicine. It involves culturing and implanting stem cells in a three-dimensional vehicle to improve the rate of cell differentiation and survival at the site of the cell graft. One of the current goals is to create a scaffold that mimics the microenvironment of the heart. This scaffold can be grafted with the cell type of choice and administered to the heart (Mazzola and Di Pasquale 2020). One of the first applications of fibrin patches was in the ESCORT trial, where patches embedded with human ESC-derived CPCs were implanted epicardially during CABG

(Menasché et al. 2018). This novel method of stem cell delivery has been validated in preclinical studies and yields high cell retention rates (Park et al. 2019). It was also reported that sheets containing MSCs could increase cell retention while amplifying paracrine effects to regenerate damaged heart tissue (Narita et al. 2013). Biomaterials such as collagen, hyaluronic acid, alginate, and a large variety of synthetic polymers have shown variable advantages and disadvantages (Mazzola and Di Pasquale 2020). Though the optimal combination of scaffolding materials and stem cells has yet to be confirmed in clinical trials, PSCs are better candidates in creating functional heart tissue since they have a more significant potential to integrate into the myocardium (Liu et al. 2018; Oikonomopoulos et al. 2018). MSCs combined with bioengineered materials will also hold a promising approach for heart repair. Currently, hydrogel-based scaffolds and cell sheet engineering are being studied (Oikonomopoulos et al. 2018). Cell sheets and scaffolds also avoid the risk of myocardial injury caused by direct injection.

Though many methods of delivery exist, the degree of cell retention within the myocardium remains very low. Though some preclinical studies have compared various routes of delivery, the results in human studies are pending. It is believed that the most effective method will likely be dependent on the type of cell in question, as all have their benefits and limitations (Turner et al. 2020). Intravenous delivery of stem cells is minimally invasive but has negligible cell retention potential. Intracoronary infusion of cells is the most commonly used method but presents a risk of coronary artery occlusion. The intramyocardial route has the greatest potential for cell retention, lacks paracrine effects, and requires skilled clinicians. The transendocardial route poses great potential for cell retention, yet is challenging to approach and requires skilled and experienced clinicians (Fig. 2). The type of stem cell, patient characteristics, and the degree of cardiac disease may all play a role in determining which route of administration is optimal for the patient in question (Nigro et al. 2018) (Table 5).

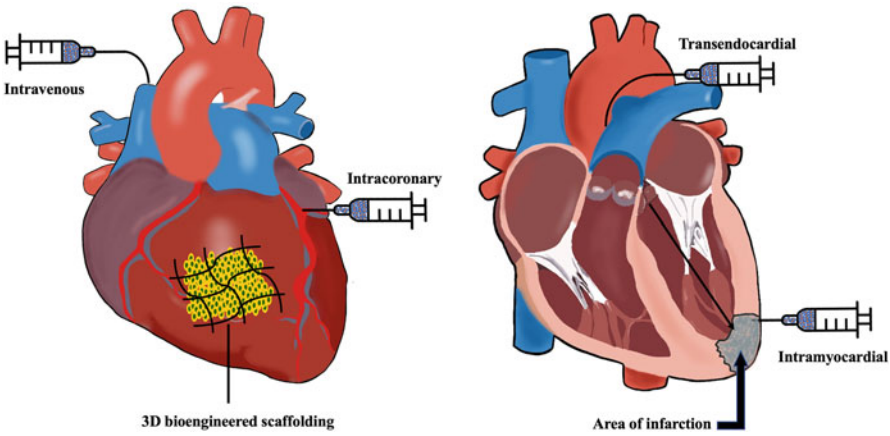

Fig. 2 Common routes of cell administration in humans

Table 5 Routes of administration: benefits and limitations

Route of administration	Benefits	Limitations
Intramyocardial	High rate of retention in the heart	• No significant paracrine effect • Invasive • Risk of perforation, arrhythmias, and emboli • Hard to distinguish between normal and infarcted tissue
Intracoronary	• Most common and safer method of delivery • Increased cell survival rates and paracrine effect	• Risk of MI with large doses • Rapid washout of cells
Intravenous	Least invasive with positive safety parameters	Lack of engraftment and retention potential
Transendocardial	• Great cell retention potential • Does not require open-heart surgery	• Difficult technique • Risk of perforation and arrhythmia formation
Bioengineering and 3D scaffolding	• High rate of cell retention • Prolongs paracrine effect • Many different biomaterials and synthetics are being tested	• Ideal combination of scaffolding and stem cells needs to be confirmed • Avoids risk of myocardial damage

Hippo-YAP Pathway

It is well established that the adult heart lacks significant endogenous regenerative potential. For this reason, researchers have focused on manipulating pathways that regulate CMs proliferation to amplify endogenous cardiac regeneration. Particular attention has been paid to the Hippo signaling pathway – an evolutionary homeostatic mechanism that controls organ size (Wang et al. 2018). The primary mechanism of this pathway is to restrict cardiomyocyte proliferation once the heart has fully developed to maintain its optimal size. This pathway also inhibits cardiac regeneration, which is problematic when tissue damage occurs. Research has focused on the Hippo-YAP (Yes-associated protein) pathway to reactivate cardiac regeneration by regulating cardiomyocyte proliferation and differentiation. YAP is a downstream effector of the Hippo pathway and is responsible for triggering transcription of cell-proliferating genes while suppressing apoptotic genes. These two characteristics are instrumental in determining regenerative potential (Xin et al. 2011). Normally, YAP is inhibited by the Hippo signaling pathway, restricting cellular growth and organ size (Wang et al. 2018). It is clear that manipulating the Hippo-YAP pathway effectively enhances fetal and neonatal cardiac proliferation and regeneration; however, the question remains if YAP activation can stimulate adult CMs' proliferation and mitigate cardiac cell death (Lin et al. 2014).

Researchers have shown that 28-day-old YAP transgenic mice post-MI had a 2.5-fold increase in cardiomyocyte proliferation, a decrease in scar size and an improvement in cardiac function compared to control mice (Xin et al. 2013). Similar results were found with the inactivation of the Hippo pathway, which led to increased DNA

synthesis and cytokinesis (Heallen et al. 2013). However, the 2.5-fold increase in CM proliferation is still 20-fold lower than the observed rate in wild-type mice. This suggests that alternative mechanisms, such as mitigation of fibrosis, reduction in apoptosis, and decreasing inflammation, may contribute a large portion of the therapeutic benefit (Xin et al. 2013). Notably, the Hippo-YAP pathway stands out from other paracrine growth factors as it promotes cardiomyocyte proliferation without the possibly deleterious effects of cardiac hypertrophy (Lin et al. 2014). More detailed studies are required to confirm the manipulation of the Hippo-YAP pathway. Nevertheless, it may be a feasible tool for treating cardiac injury and triggering endogenous cardiac regeneration in humans.

Ethical Issues in Regenerative Medicine

In an attempt to lower the global burden of cardiovascular diseases, stem cells have quickly gained momentum in research and are being explored at an unprecedented rate. However, their use generates various ethical and political issues not commonly seen in other treatment modalities. The most prominent ethical issue arises from the morality of using human embryonic stem cells in research. The creation of a human ESC line involves the extraction of the inner cell mass from the blastocyst during 5–7 days of fetal development, which results in the destruction of the human embryo and the potential for human life. This ethical dilemma begs the question of when does human life truly begin. Some believe that human life begins at conception, whereas others believe that it begins further into development or even at birth (Lo and Parham 2009). A current strategy to circumvent this issue involves only using embryos that have initially been produced for reproductive purposes. Although this may ease the ethical burden for some, it is a near-impossible task appeasing all the parties involved in the matter. Alternatively, the use of other stem cell types eliminates the ethical concerns regarding the destruction of potential human life. Currently, iPSCs are seen as an attractive alternative to ESCs. They are derived from adult stem cells and genetically programmed back to a higher state of potency, making them a strong contender to the ESC. Adult stem cells can also be viable options, but their use is limited, and they lack the level of potency seen in ESCs and iPSCs (Barile et al. 2007).

Another concern regarding the use of stem cells is the growing trend of "stem cell tourism," which describes the practice of patients seeking expensive, unproven stem cell treatments at private clinics around the world. These treatments are frequently promoted as a definite cure; however, they often lack sufficient data on clinical efficacy or safety (Ryan et al. 2010). This dangerous trend has resulted in the opening of unregulated clinics that exploit their patients for their profits, sometimes charging fees of $30,000 for unproven treatments (Regenberg et al. 2009). Unfortunately, the public is often ill-equipped to gauge whether or not treatments offered in clinics are safe and credible, and only 29% of clinics that offer stem cell treatments are accredited to do so (Connolly et al. 2014). Although the applications of stem cells are pretty exciting, cardiac regeneration is still a relatively novel concept.

Particularly for HF treatment, many variables must be accounted for before stem cells can make their way into medical practices. It is imperative that scientific standards and methodologies remain at the highest degree to avoid breaches of scientific integrity, as previously seen in the study of CSCs.

What Does the Future Hold for Cardiac Regenerative Medicine?

Cell-Free Strategies

Data from preclinical and clinical trials seems to come to a reasonably consistent conclusion that low cellular retention rates and the inability to generate new CMs adequately remain a concern with current cellular strategies (Maghin et al. 2020). In addition, it postulated that a large portion of the therapeutic benefit of stem cells is derived from the biological factors that they secrete rather than direct cellular differentiation (Park et al. 2018). Recently, much attention has been paid to utilizing a cell-free approach to cardiac regeneration via administering a "cocktail" of cardioprotective paracrine signaling molecules. This combination of factors secreted by progenitor or stem cell populations includes cytokines, growth factors, and miRNAs and has been termed the "secretome." Several strategies to deliver these paracrine factors include direct administration of individual growth factors, use of endogenous extracellular vesicles secreted by various cell types, delivery of cultured medium of stem cells, and the overexpression of proteins via modified mRNA (Liew et al. 2020).

One of the first studies that truly corroborated the validity of the paracrine hypothesis was the use of a conditioned medium of cultured human MSCs into a porcine model of acute MI (Timmers et al. 2007). It was demonstrated that a single IC injection of human MSC cultured medium into an ischemic porcine model was associated with a 60% reduction in infarct size and improvement in both systolic and diastolic cardiac function (Timmers et al. 2007). Precisely, it is thought that small EVs or exosomes may contribute a significant portion of the stem cell secretome's regenerative effects (Kishore and Khan 2016). EVs is a collective term that describes small phospholipid MVs secreted by cells and contain biologically active compounds such as proteins, growth factors, RNA, and biolipids (Simons and Raposo 2009). Although they may be used interchangeably in the literature, exosomes refer to a subclass of extracellular vesicles that range from 40 to 100 nm and originate as endosomes (Simons and Raposo 2009). However, the source of extracellular vesicles is not limited to MSCs. A study was published delivering exosomes derived from CDCs into a porcine model of acute MI (Gallet et al. 2017). The pigs received either IC or open-chest IM injection of either placebo vehicle or exosomes 4 weeks post-MI. They demonstrated that pigs receiving CDC-derived exosomes had a preserved LV volume and LVEF, as well as histologic improvements in vessel density and cardiomyocyte hypertrophy.

Interestingly, a separate study investigated the efficacy of a mixture of human iPSC-derived CMs, endothelial cells, and smooth muscle cells versus only exosomes

extracted from these PSC-derived cell types (Gao et al. 2018). It was found that cardiac outcomes such as LV function, angiogenesis, infarct size, and wall stress were similar in both the exosome group and cell group, and both were significantly improved compared to MI without treatment group. Moreover, exosome therapy did not increase the frequency of arrhythmia, a primary concern from studies investigating human iPSC-derived CMs in a primate model (Shiba et al. 2016; Romagnuolo et al. 2019). These findings suggest that exosomes alone could potentially be as effective as stem cells in the treatment of cardiac pathologies. However, human clinical trials will need to be completed on exosome use before these conclusions can be definitively made.

The direct administration of cellular growth factors appears to be the most simplistic strategy for delivering paracrine factors to the heart. Two standard strategies to induce growth factor gene expression include the direct injection of recombinant proteins or the administration of viral vectors or plasmids containing the growth factor encoded gene (Spannbauer et al. 2020). Most preclinical studies have focused on several key growth factors: vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), although many others have also been investigated (Liew et al. 2020). A similar strategy revolves around using RNA, including coding messenger (mRNA) and noncoding RNA (ncRNA). One of the first studies that demonstrated this concept involved the epicardial or IC administration of VEGF and effectively improved myocardial blood flow and enhanced regional ventricular function in a porcine model of chronic ischemia (Lopez et al. 1998). In another study, porcine models of chronic ischemic heart disease were co-transfected with VEGF-A and platelet-derived growth factor B (PDGF-B) plasmids (Kupatt et al. 2010). The pigs co-transfected with VEGF-A and PDGF-B showed significant neovascularization and improved regional and global myocardial function. In gene therapy, there have been over 150 human clinical trials investigating the effects of various therapeutic molecules on cardiac angiogenesis (Cannatà et al. 2020).

However, there remains no gene therapy that has been proven to be successful in achieving clinical benefit. There have also been several landmark clinical trials that specifically investigated the use of gene therapy in the treatment of HF. The main targets in these trials were various components of the calcium handling system within CMs (Cannatà et al. 2020). The first key clinical trial was the phase 1/2 CUPID trial, which aimed to restore the sarcoplasmic Ca2+ ATPase (SERCA2a) in 39 patients with HF (Jaski et al. 2009; Jessup et al. 2011). The SERCA2a gene was delivered via cDNA in an adenovirus vector (AAV-1) during a single IC infusion. Patients were stratified into a low dose, medium dose, high dose, or placebo group. Initial results were promising, as a positive safety profile was confirmed, and there was a trend toward symptomatic improvement and a significant reduction in cardiovascular events at 12 months (Jessup et al. 2011). This prompted a follow-up with the much larger randomized, placebo-controlled phase II CUPID2 clinical trial, which enrolled 250 patients with HF (Greenberg et al. 2016). Unfortunately, results were disappointing as patients given AAV-1/SERCA2a did not have an improvement in cardiovascular events. A similar story can be told with a different target, the stromal cell-derived factor-1 (SDF-1). The SDF-1 growth factor promotes cell

survival and recruitment, triggers angiogenesis, and increases tissue repair (Chung et al. 2015).

Initial phase I clinical trials tested the safety and efficacy of an endomyocardial injection of a DNA plasmid encoding SDF-1 into 19 patients with ischemic cardiomyopathy (Penn et al. 2013). The trial demonstrated a positive safety profile and qualitative symptomatic improvement. However, the larger, randomized phase II STOP-HF trial fell short and could not reproduce the positive results seen in the earlier trials (Chung et al. 2015). However, this does not mean that all hope is lost as several other targets have either shown initial promise or are currently in clinical trials (Hulot et al. 2016). Interestingly, an ongoing phase I clinical trial investigates the combination of VEGF-A, SDF-1 alpha, and S100 calcium-binding protein A1 as triple gene therapy in patients with end-stage HF and an implantable left ventricular assist device (NCT03409627). The ongoing EPICCURE study will be the first clinical trial to administer mRNA into the human heart (NCT03370887). It aims to investigate the safety and efficacy of AZD8601, a VEGF-A mRNA formulated in an injectable saline solution. Twenty-four patients with moderately decreased EF will be given a round of 30 epicardial injections of either 30 mg, 3 mg AZD8061, or placebo during a CABG procedure and followed for 6 months.

Cell-free strategies appear to overcome potential concerns surrounding conventional stem cell therapies, particularly from a translational perspective. These include safety concerns such as immune compatibility and rejection, as well as practical concerns such as accessibility, cost-effectiveness, and time-consuming procedures (Maghin et al. 2020). The necessary next steps include determining the optimal route of administration of these factors. In summary, noncellular regenerative therapies such as single growth factors, RNAs, or combination therapies such as exosomes appear to have significant myocardial reparative capacity. Exosomes circumvent many practical issues such as cell retention and immunogenicity and could potentially serve as an alternative to the cell-based approach in the future.

Bioengineering

The lack of understanding of the optimal cell type, dosage, route of administration compounded by low cell retention and survival, poor engraftment, and ineffective differentiation of progenitor cells post-transplantation are some of the most significant barriers encountered in clinical trials. Tissue engineering may hold the key to improving cell delivery and retention. The extracellular matrix (ECM) is now understood to be a dynamic microenvironment that contains complex networks of various proteins and growth factors and provides physical and mechanical signals to modulate cell behavior and differentiation (Maghin et al. 2020). Two strategies to bioengineer a matrix include creating a synthetic microenvironment or extracting ECM from tissues and decellularizing the matrix to create a cell-free product (Domenech et al. 2016). Since the cell microenvironment plays such an essential role in cell behavior, researchers have aimed at creating constructs that mimic the environment to improve cell culture systems and improve effectiveness of stem cell

transplantation. In the last several years, 3D culture systems have improved differentiation techniques to create cell phenotypes similar to those in native tissues (Mazzola and Di Pasquale 2020). Several studies have demonstrated that differentiation of human PSCs or iPSCs-CMs in 3D culture systems improved the level of cell maturation (Ronaldson-Bouchard et al. 2018; Correia et al. 2018). Ronaldson-Bouchard et al. (2018) showed that early iPSCs-derived CMs cultured on a 3D hydrogel subjected to electrical and mechanical stimulation showed a similar gene expression profile, sarcomere length, density of mitochondria, and functional calcium handling similar to mature adult cells.

A separate strategy in tissue engineering revolves around implanting the bioengineered tissue construct with stem cells or biologically active molecules, which is believed to increase cellular retention rates and lengthen the duration of regenerative paracrine signaling (Micheu 2019). Tissue constructs known as cardiac patches were created by adding stem cells to natural or synthetic biomaterials such as fibrin, collagen, alginate, or even a natural decellularized ECM and mimicking the biomechanical or electrical signaling they would receive in vivo (Mazzola and Di Pasquale 2020). These cardiac patches can be directly transplanted onto the epicardial surface of the heart and serve as a temporary scaffold to enable cell engraftment into the host heart. Thus, the choice of biomaterial for the scaffold is important in determining the functional survival, engraftment, and proliferation of implanted cells (Mazzola and Di Pasquale 2020). Preclinical trials have shown that administration of cells embedded in patch construct improved cellular engraftment and decreased the number of cells needed for the same graft size and functional benefit by tenfold compared to cell injection studies (Weinberger and Eschenhagen 2021). Efficacy and safety have also been demonstrated in a porcine model of MI. Researchers showed that a cardiac muscle patch produced from human iPSC-derived CMs, smooth muscle cells, and endothelial cells loaded onto a fibrin scaffold induced significant improvements in LV function, infarct size, and protective effects on endogenous CMs (Gao et al. 2018). Since tissue engineering is a relatively novel concept in cardiac regeneration, there have been relatively few clinical trials investigating the use of cardiac scaffolds. The groundbreaking ESCORT trial discussed earlier demonstrated the effective and safe use of a fibrin patch embedded with human ESC-derived CPCs (Menasché et al. 2018).

Likewise, a recent clinical trial out of Japan compared the use of human umbilical cord mesenchymal stromal cells embedded in a collagen hydrogel versus cell treatment only in patients with chronic ischemic heart disease (NCT02635464). Fifty patients were randomized into either cell/hydrogel group, cell only, or placebo, and were given a single IM injection during a CABG procedure. Results showed that the collagen hydrogel treatment was a safe and feasible delivery option, and the collagen/cell combination decreased mean scar size at 12 months. However, results were not statistically significant (He et al. 2020). However, improvements in LVEF, NYHA functional class, viable heart mass, and quality of life measured by the MLHFQ were noted. Moreover, LVESV and LVEDV decreased. This study, to our knowledge, is the first ever to establish that the use of collagen hydrogel in humans is safe and feasible for cell delivery. These findings will provide a basis for future clinical trials in the future.

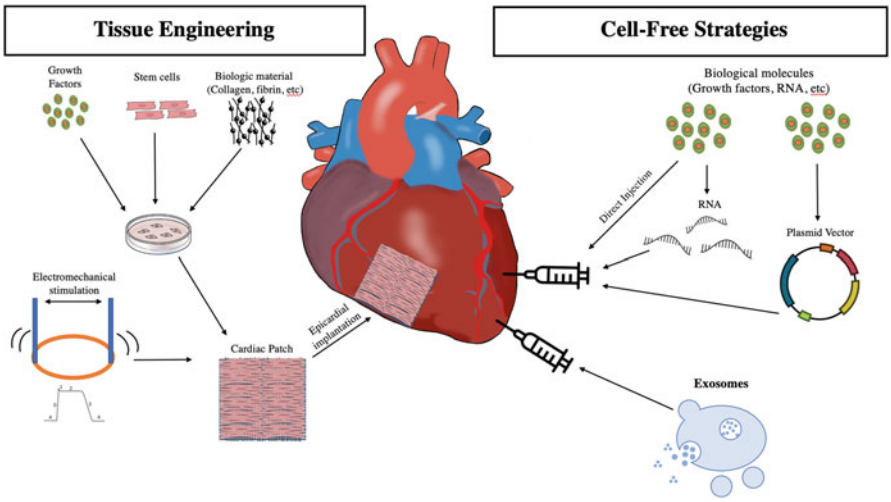

Fig. 3 Approaches to tissue engineering and cell-free strategies in cardiac regeneration

Recently, researchers have created scaffold-free cell sheets which can be directly implanted onto the myocardium without transplantation of a biomaterial in addition to the cells. This technology involves culturing a monolayer or multilayer of cells on a thermoresponsive polymer surface, and then removing the individual sheets of cells to combine them and create 3D cardiac grafts (Zhang 2015). However, this recent phenomenon has been well proven in various preclinical animal models but has yet to be tested in the clinical trial setting. Because of the low rates of cellular retention and cardiac remuscularization involved with direct cell injection, alternative methods needed to improve cardiac regeneration. Many believe that combination therapy holds the key to improving the efficacy of cardiac regeneration through enhanced cell engraftment and also improved paracrine factor signaling. This likely involves not only the combination of cells embedded in scaffolds, but also the involvement of biologically active molecules such as growth factors, RNAs, and exosomes. It seems inevitable that future research will investigate the implantation of a cardiac construct containing several different cell types and signaling molecules as a cell sheet or embedded in a biologically active scaffold (Fig. 3).

Conclusion and Future Perspectives

As the field of cardiac cell therapy approaches its third decade, we have yet to have a single stem cell type that the FDA approves for the treatment of heart disease (Cingolani 2019). However, it appears that we are finally gaining a greater understanding of the issues that have plagued the discipline for so long. Through a plethora of clinical trials, we have witnessed that it is incredibly difficult to remuscularize the failing heart. Cellular retention and survival rates have been

incredibly low in clinical trials. In response, the field has shifted its mechanistic hypothesis of how cardiac cell therapy provides the observed therapeutic benefits. Most researchers now believe that stem cells provide the vast majority of their cardiac benefit via paracrine signaling rather than directly producing new CMs to remuscularize the heart. However, there appears to be some variation between specific cell types and method of delivery.

Thus far, numerous clinical trials have shown that cardiac cell therapy generally has a favorable safety profile. The SafeCell Heart meta-analysis demonstrated no adverse events associated with the use of MSCs in heart disease (Lalu et al. 2018). There are concerns surrounding the arrhythmogenicity of stem cells primarily within the skeletal myoblast lineage. Nonetheless, all cell types should be thoroughly investigated for arrhythmogenic risk before applied in large-scale clinical settings (Chen et al. 2020).

Most clinical trials to date have demonstrated neutral to marginally positive results in terms of clinical efficacy. Interestingly, a trial sequential analysis in 2016 revealed that there is firm evidence supporting that bone marrow-derived stem cell therapies do reduce the risk of rehospitalization and mortality in patients with HF (Fisher et al. 2016b). The SafeCell Heart meta-analysis also demonstrated that patients who receive MSCs therapy have a significantly improved LVEF (Lalu et al. 2018). Nonetheless, there has only ever been one phase III clinical trial investigating cellular therapy in heart disease and the results are still pending. This does not imply that stem cells have no clinical value in heart disease. Rather, we have not found the optimal cell type or delivery system for these stem cells. Large comparative clinical trials will need to be performed before reaching any conclusions about the effectiveness of regenerative therapies compared to our conventional pharmacologic treatments currently used for HF.

Clinical research to date has set the foundations for a technological breakthrough in cardiac cell therapy. In particular, we believe that future research in three specific areas will produce a generational breakthrough in cardiac regeneration. First, cell-free sources of cardiac regeneration such as exosomes, growth factors, and RNAs may provide a potential therapeutic approach without some of the safety concerns associated with cellular therapies. A cell-free strategy may also have practical advantages in terms of scalability, availability, and reduced cost. Second, combining cell therapy with bioengineering scaffolds or tissue constructs may provide a way to increase cell engraftment rates and prolong the survival of cells so that their beneficial paracrine effects can be sustained for longer. Lastly, the use of pluripotent cells to derive cell phenotypes of choice could be an excellent strategy for creating functional CMs or endothelial cells that can directly engraft into the myocardium and synchronize with the host cells. In addition, combination therapy of multiple PSC-derived cells types has already been proven in clinical trials to provide successful cardiac outcomes.

In summary, future research must establish the optimal cell type, route of delivery, and dosage to improve the efficacy to levels needed for implementation at the clinical level. In addition, studies should have long-term follow-up periods to truly identify the therapeutic risks and potential of cardiac cell therapy. Tailoring the cell

therapy to the patient in question would be likely the most productive approach, as many differences exist between patients such as age, the severity of HF, immune status, and comorbidities. In addition, combination approaches consisting of pharmacological, cell-free paracrine strategies, and stem cell therapy will likely provide superior, sustainable results for patients. Regenerative therapies are still far from being implemented as a mainstay in the clinic; however, incredible progress has been in the field in the last several years. We remain cautiously optimistic that innovative techniques such as bioengineering, exosome therapy, and combination therapies will propel the field into unprecedented territory in the years to come.

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Therapeutic Uses of Stem Cells for Heart Failure: Hype or Hope

17

Mathieu Rheault-Henry, Ian White, and Rony Atoui

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Abstract

Heart failure is one of the leading causes of morbidity and mortality globally. Myocardial infarction remains one of the primary causes of chronic heart failure due to cardiac remodeling, including the formation of fibrotic scar tissue and cardiomyocyte necrosis. Despite myocardial infarction being recognized as a significant predisposing factor to heart failure, modern therapies such as pharmacological and surgical interventions are currently unable to treat the underlying

M. Rheault-Henry · I. White · R. Atoui (✉)
Division of Cardiac Surgery, Health Sciences North, Northern Ontario School of Medicine, Sudbury, ON, Canada
e-mail: mrheaulthenry@nosm.ca; iwhite@nosm.ca

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pathologies. Because of the limited intrinsic regenerative capacity within the heart, stem cell therapy has recently been touted as a potential option for the treatment of heart failure. Stem cells are characterized by two unique properties; the ability to self-renew and to differentiate into multiple lineages. Although the exact regenerative mechanism remains relatively unknown, it is believed that stem cells mediate myocardial repair primarily via activation of paracrine signaling pathways, with some cell types contributing to the direct replacement of functional cardiomyocytes. Pre-clinical and clinical studies have shown a range of predominantly neutral to moderately positive outcomes in terms of both safety and clinical efficacy. The following review will discuss what is currently known about stem cell-based treatment for heart failure, the various types of stem cells used in therapeutics, recent clinical trials, as well as current limitations and future directions of regenerative cell therapy.

Keywords

Adult stem cells · Bone marrow stem cells · Cardiomyocytes · Cardiovascular · Cell therapy · Chronic cardiomyopathy · Heart failure · Myocardial infarction · Pluripotent stem cells · Regenerative therapy · Stem cell

Abbreviations

ABMCs	Autologous bone marrow cells
AMI	Acute myocardial infarction
BMDSCs	Bone marrow derived stem cells
BMMNCs	Bone marrow mononuclear cells
CABG	Coronary artery bypass grafting
CPCs	Cardiac progenitor cells
CSCs	Cardiac stem cells
CVD	Cardiovascular disease
EDV	End diastolic volume
ESCs	Embryonic stem cells
ESV	End systolic volume
HF	Heart failure
HFpEF	HF with preserved ejection fraction
HFrEF	HF with reduced ejection fraction
IGF-1	Insulin-like growth factor-1
iPSCs	Induced pluripotent stem cells
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
MSCs	Mesenchymal stem cells
PSCs	Pluripotent stem cells
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Introduction

Cardiovascular disease (CVD) continues to be the leading cause of morbidity and mortality globally, with the World Health Organization (WHO) reporting an estimated 17.9 million deaths attributed to CVD in 2016 (World Health Organization 2021). Chronic heart failure (HF) is an important cardiovascular disease due to its high incidence, high mortality rate, and poor prognosis (Alpert et al. 2017; Hong-Mi et al. 2019). HF is a debilitating disease that leads to progressive cardiopulmonary symptoms and is strongly associated with a decreased quality of life (Merck Manuals Professional Edition 2020). HF currently affects 1.5% of Canadians (Tran et al. 2016) and these numbers are expected to increase due to the aging demographic and the increase in CV risk factors such as obesity. Canadians who are hospitalized with HF have a 1-year mortality rate of over 30% and these numbers have not improved over the last decade despite significant medical advancements (Ibid.).

Pathophysiology of Heart Failure

Heart failure is a complex syndrome of ventricular dysfunction that results from structural or functional impairment of ventricular filling or ejection of blood. This subsequently results in inadequate blood supply to the tissues for metabolic needs (Merck Manuals Professional Edition 2020). The most common underlying cause of HF is myocardial infarction (MI) (Cahill and Kharbanda 2017), which is defined as myocardial cell death due to prolonged ischemia (Thygesen et al. 2012). However, HF may also be caused by structural defects like congenital deformities, valvular disorders (i.e., aortic stenosis), high metabolic demand (i.e., thyrotoxicosis), myocarditis, arrhythmias and persistently high heart rate (Merck Manuals Professional Edition 2020). In MI, single or multiple coronary arteries are occluded persistently or intermittently, which impedes perfusion of the myocardium (Thygesen et al. 2012). After the onset of ischemia, cardiomyocyte death is observed and an intense inflammatory response is triggered (American College of Cardiology 2016). Besides, reperfusion of the myocardium causes the production of reactive oxygen species, release of vasoactive mediators, and plugging of inflammatory cells leading to further myocardial damage (Cahill and Kharbanda 2017). In the post-MI state, there is a progression from inflammation to cardiac repair, including the formation of fibrotic scar tissue, activation of the sympathetic nervous system and initiation of the renin-angiotensin-aldosterone system (Braunwald 2013). Cardiac remodeling results in structural and functional changes in the zone of infarction (Merck Manuals Professional Edition 2018) and can ultimately lead to HF. The most common classifications of HF are HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF) (Yancy et al. 2013). HFrEF is defined by HF with left ventricular ejection fraction (LVEF) of $\leq$40%. In this type of HF, the ventricle fails to contract properly, leading to increased diastolic volume and

diastolic pressure with decreased ejection fraction. Over time, the ventricles are remodeled as a higher preload is required to maintain cardiac output. As the ventricle dilates and hypertrophies, increased stiffness of the ventricle and diastolic dysfunction ensues, compromising cardiac performance, and further contributing to adverse systemic venous congestion (Merck Manuals Professional Edition 2020). Conversely, HFpEF is defined as HF with LVEF ($\geq$ 50%). In this case, the ventricular filling is impaired as the heart is unable to relax and becomes stiff. Increased ventricular end-diastolic pressure and normal end-diastolic volume, ejection fraction, and contractility are observed (Yancy et al. 2013). HFrEF and HFpEF can affect both ventricles, though the left ventricle is most commonly affected. A deep understanding of the pathophysiology of HF is critical in producing an effective stem cell-based strategy that reverses underlying pathological processes.

Significance to the Research

Despite tremendous medical advancements in the past generation, the diagnosis of chronic heart failure still carries significant morbidity and mortality (Kostuk 2001). Current goals of HF treatment are aimed at alleviating symptoms, properly managing comorbidities, and enhancing the quality of life rather than reversing the underlying pathologic process. Standard pharmaceutical therapies involve medication such as diuretics, beta-adrenergic blockers, anti-hypertensives, inotropic agents, and anticoagulants (Inamdar and Inamdar 2016). HF caused by MI can be treated with surgical intervention to relieve obstructed arteries and reperfuse the myocardium (Briffa et al. 2009). Smoking cessation, healthy dietary patterns, regular physical activity, and maintaining a normal body mass index (BMI) are common risk reduction techniques (Fleg 2016) that serve to slow disease progression without reversing pre-existing damage to the heart (Faiella and Atoui 2016). Heart transplantation is generally used as a last resort for all-cause HF (Yamakawa et al. 2013) and carries a 10-year survival rate of over 50% (Anyanwu and Treasure 2003). However, there remains a major discrepancy between the availability of donors and recipients (Ibid.). Research is now focusing on promising alternative therapy to treat HF: stem cells. It is believed that stem cells can regenerate damaged cardiac tissue and restore its physiologic function (Fraser et al. 2004). The following review will discuss the advantages and limitations of various stem cell therapies, including current clinical trials, safety concerns, and how to optimize cell therapies.

Introduction to Stem Cells

Stem cells are characterized by two unique properties: the ability to self-renew and to differentiate into specialized cell types. Although first identified in the hematopoietic system, various subtypes are present in other tissues, which have led to the development of multiple system-specific therapies. They are gaining popularity and their roles have been evolving within the following three major applications in medicine.

1. Cell therapy to replace functional tissue
2. Targets of drug therapy
3. To generate differentiated tissue for in vitro study of disease models to develop drugs

Stem cells have varying differentiating potentials, known as potency. Potency is the capacity of the stem cell to differentiate into specialized cell types (Hima and Srilatha 2011) which will give rise to a mature cell type and, subsequently, the formation of tissues within the body. Once an egg is fertilized in the female, the cells that arise in the first few divisions are classified as totipotent, meaning that they can generate a viable embryo. However, within a matter of days, these totipotent cells transition to pluripotent, subsequently becoming embryonic stem cells (ESCs). The level of their potency decreases as stem cells differentiate further along their lineage pathways. Thus, stem cells can be classified into three clinically relevant categories based on their origin and level of potency: ESCs, induced pluripotent stem cells (iPSCs), and adult stem cells.

Embryonic Stem Cells

ESCs are a descendant of totipotent cells. These cells are derived from the inner cell mass of the blastocyst, a hollow ball of cells that forms 3–5 days after an egg is fertilized (Stem Cells 2020). ESCs are pluripotent, meaning that they have the ability to produce cells from all three germ layers (ectoderm, mesoderm, endoderm), but not the placenta or umbilical cord. These cells are a resource for studying normal development, disease, and for testing drugs and therapies (Ibid.). Current therapeutic strategies involve differentiating ESCs into the tissue-specific cell or progenitor before incorporation into the tissue of choice. When ESC lines are produced in the laboratory, they retain their proliferative properties indefinitely. These are invaluable since they have the highest differentiation potential but are subject to various ethical debates (Hima and Srilatha 2011) (discussed in Ethical Considerations of the Use of Stem Cells).

Induced Pluripotent Stem Cells

iPSCs- have been engineered in laboratories by converting tissue-specific somatic cells into stem cells that behave like ESCs (Stem Cells 2020). For example, isolating keratinocytes from the epithelium and engineering it into a pluripotent stem cell with the capacity to differentiate into all three germ layers. Like ESCs, these can help researchers to learn more about normal development, characterize the disease, and investigate novel therapeutics (Ibid.). Although these cells share many of the same characteristics of ESCs, several important differences distinguish them from ESCs. One of the major advantages of iPSCs is that they circumvent serious ethical concerns that come with ESCs. However, it is believed that iPSCs retain some

residual epigenetic memory of their specialized cell of origin (Kim et al. 2010), making it difficult to effectively reprogram to a fully pluripotent phenotype. As with ESCs, current strategies of cardiac therapeutics involve "instructing" iPSCs to differentiate into cardiac progenitor cells (CPCs) or cardiomyocytes before implantation into the myocardium.

Adult Stem Cells

Tissue-specific stem cells are commonly referred to as adult stem cells. These are more specialized stem cells (Hima and Srilatha 2011) and can only differentiate into a limited number of cell types within their tissue of origin. Tissue-specific stem cells are classified as multipotent, but their products are unipotent as they are of the lowest differentiation potential. For example, hematopoietic stem cells are multipotent, but red blood cells are unipotent as they are at their last stage of differentiation. The adult stem cells of interest in this discussion are cardiac stem cells (CSCs), bone marrow-derived stem cells (BMDSCs), and mesenchymal stem cells (MSCs). CSCs are isolated from the atria, ventricles, or the epicardium in biopsies (Faiella and Atoui 2016) and play an important role in clinical trials. Although they are harder to acquire and possess less impressive self-renewal capabilities, they have many attributes that improve cardiac function (Menasché 2018). The only CSCs used in clinical trials are KIT+ and cardiosphere-derived cells. Bone marrow-derived mononuclear stem cells (BMMNCs) are adult stem cells that contain various populations of hematopoietic and immune cells. Lastly, MSCs are commonly mentioned in stem cell research. Many tissues in the body contain these multipotent cells, the largest reservoir being in the bone marrow (International Society for Stem Cell Research). These fall under the category of adult stem cells, and although found in the bone marrow, they are classified under their subtype. These cells are capable of forming bone, cartilage, and fat cells (Ibid.). Although these fall under tissue-specific stem cells, they are classified under their own lineage.

Endogenous Regeneration of Cardiomyocytes

Heart regeneration has been a heavily investigated topic in a countless number of species. In the developing mammals and invertebrates, there is well-documented evidence showing vast myocardial regeneration (Laflamme and Murry 2011). Yet, the extent of endogenous myocardial regeneration in adult hearts is still up for debate. The vast majority of the growth of the human embryonic and fetal heart occurs due to the rapid proliferation and differentiation of CPCs and immature cardiomyocytes (Eschenhagen et al. 2017; Eldad et al. 2017). Throughout the postnatal life, the central mechanism for cardiac growth shifts from hyperplasia to cardiomyocyte hypertrophy (Eschenhagen et al. 2017). One current theory postulate that the polyploid genome found in adult human cardiomyocytes restricts regeneration. In contrast, animal cardiomyocytes such as amphibians and reptiles have an

almost entirely diploid genome (Marchianò and Murry 2019) allowing cells to divide more frequently. Further investigations suggest that polyploidy is strongly correlated with endothermy, the ability to regulate one's body temperature. As one's body temperature and metabolic rate increase, there is a decrease in diploid cardiomyocytes, leading researchers to think that there may have been an evolutionary trade-off between the ability of humans to regenerate cardiac tissue and the ability to regulate body temperature (Ibid.). Nonetheless, one thing is for certain: the human heart is not a completely post-mitotic organ (Bruyneel et al. 2016). Current evidence estimates that in a healthy human adult heart, the cardiomyocyte turnover rate appears to be around 1% per year, with a decline in turnover rate with age (Burridge et al. 2012; Bergmann et al. 2009). This equates to approximately 50% of cardiomyocytes being renewed across one's lifespan (Bergmann et al. 2009). This can become incredibly problematic, given that a severe myocardial infarction can destroy 25% of functional cardiomyocytes within only a few hours (Murry et al. 2006). Interestingly, several studies suggest that the myocardium may have some innate proliferative ability after a severe cardiac event. In 2001, Beltrami et al. discovered that there was an increase in human cardiomyocyte renewal post-myocardial infarction in the border regions of the infarct. However, it is uncertain if this provides any clinical benefit. The question remains as to where these cardiomyocytes arise. The majority of studies seem to suggest that the proliferation of existing cardiomyocytes provides the largest contribution to basal cardiomyocyte turnover, with differentiation of resident and extracardiac stem cells occurring at a higher rate after injury (Eschenhagen et al. 2017). A deeper understanding of the exact mechanisms underpinning endogenous cardiomyocyte regeneration after myocardial injury would be extremely beneficial for developing the most effective therapeutic strategy.

Landmark Clinical Trials

Adult Stem Cells

Cardiac Stem Cells

Until recently, it was controversial as to whether CSCs had the potential to regenerate the myocardium. However, it is now well established that CSCs express markers of cardiogenesis and can differentiate into cardiomyocytes and vascular endothelial cells (Faiella and Atoui 2016). To date, only c-KIT+ CSCs and cardiosphere-derived cell phenotypes have been utilized in clinical trials. The stem cell infusion in patients with ischemic cardiomyopathy (SCIPIO) is a phase 1 clinical trial (2011) administering KIT+ CSCs to patients with HF undergoing coronary artery bypass grafting (CABG). Interestingly, KIT+ cells are speculated to favor differentiation into vascular cells rather than cardiomyocytes (van Berlo et al. 2014). Preliminary results of the study demonstrated an increase in LVEF at 4- and 12-months post-infusion, in addition to a decrease in infarct size (Chugh et al. 2012). However, in 2014 the editors of The Lancet issued an "expression of

concern" (Menasché 2018) as the integrity of the data was questioned, leading to the retraction of the study. The intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS) trial is a published phase 1 clinical trial examining the use of autologous cardiosphere-derived CSCs. Six months post-administration, MRI findings showed a reduction in scar tissue size, along with improvements in regional contractility and viable heart mass. Although structural and functional changes were noted, changes in end-diastolic volume (EDV), end-systolic volume (ESV), and LVEF did not differ between groups (Makkar et al. 2012). There were no significant adverse effects noted in the SCIPIO and the CADUCEUS trials, alluding to a positive safety profile for CSCs. The CONCERT-HF trial is an ongoing trial including 144 patients with HF who are assigned randomly to receive KIT+ CSCs, bone marrow-derived MSCs, a combination of both cell types or a placebo. The premise of using both cell types together is based on animal models showing that the pairing works synergistically (Bolli et al. 2018) to improve structural and functional outcomes in chronic HF. Although this study was paused for a brief period, it is set to be completed by July 2020 (National Institute of Health, n.d.). Likewise, the TAC-HFT-II trial will soon compare therapy with autologous MSCs alone versus MSCs combined with KIT+ CSCs. The ALLSTAR phase 2 trial using cardiosphere-derived cells was stopped prematurely due to the futility of the data and the probability of not meeting the primary goal after 6 months follow-up (Menasché 2018).

Bone Marrow-Derived Cells

Bone marrow-derived stem cells (BMDSC) have been one of the most heavily tested cell types in the treatment of cardiovascular disease to date. However, many of these trials focus on acute myocardial infarction (AMI) rather than HF (Turner et al. 2020). While MSCs are often isolated from the bone marrow, this section will be focusing on BMMNCs and autologous bone marrow cells (ABMCs). The first-ever clinical trial using cellular therapeutics was published in 2003. It included 21 patients with chronic ischemic cardiomyopathy who received transendocardial injection of autologous BMMNCs. After 4 months, there was a significant increase in LVEF and a reduction in ESV (Perin et al. 2003). Similar results were found in the TOPCARE-CHD trial, which showed a significant decrease in brain natriuretic peptide and decreased mortality in response to intracoronary administration of BMMNCs (Assmus et al. 2007). The STAR-heart study demonstrated that up to 5 years after intracoronary administration, ABMCs improved long-term mortality and LVEF (Tehzeeb et al. 2019). Besides, a decreased left ventricular preload, end-systolic volume, systolic wall stress, occurrence of arrhythmias, and area of infarction was noted. To this point, all clinical trials had also demonstrated a positive safety profile for BMDSCs. This initial success set the stage for the larger phase 2, randomized, double-blind FOCUS-CCTRN trial. This trial enrolled 153 patients with chronic ischemic cardiomyopathy and aimed at administering autologous BMMNCs via transendocardial injection. Unfortunately, these positive results could not be replicated, as there were no significant improvements in LVEF,

maximal oxygen consumption, or infarct size (Perin et al. 2012). Additionally, In the TAC-HFT-I trial, patients were given either transendocardial injections of autologous BMMNCs, autologous MSCs, or placebo. Results showed that only MSC therapy decreased infarct size and improved regional function of the heart, and the distance walked during the 6-min walk test (Heldman et al. 2014). No improvements were noted in LVEF. After more than a decade of research, a trial sequential analysis of two Cochrane reviews was published, providing clarity as to the overall effectiveness of BMDSCs in the treatment of HF. They determined that there is firm evidence showing BMDSCs cause a reduction in both mortality and rehospitalization rates. However, they show that treatments do not provide more than a 4% increase in LVEF (Fisher et al. 2016a). There is also a large phase 3 clinical trial ongoing to assess if the intracoronary infusion of autologous BMMNCs is safe and can cause a reduction in mortality and LVEF. If positive data comes out of the ongoing BAMI trial, we may very well see a public push for autologous BMMNCs to transition from bench to bedside.

Mesenchymal Stem Cells

Preclinical and clinical evidence suggests that MSCs may provide benefit in the treatment of MI and HF (Lalu et al. 2018) due to a greater likelihood of vascular proliferation and regeneration (Tehzeeb et al. 2019). MSCs exhibit important reparative properties such as immunomodulation, along with antifibrotic, proangiogenic, and anti-oxidative effects (Turner et al. 2020) making them great contenders for treating cardiomyopathies such as HF. By March 2018, there were 17 clinical trials registered using MSC therapy in chronic HF. Among the different bone marrow-derived cells, MSCs seem to show the greatest promise for regeneration of myocardium (Menasché 2018), likely due to their strong paracrine effect. The POSEIDON randomized control trial compared autologous and allogeneic MSCs in HF patients. Results indicate that both types of cells reduced infarct size, adverse cardiac remodeling, and LV function after an MI (Hare et al. 2012). A recent systematic review and meta-analysis which included 23 studies in total, investigated the safety and efficacy of adult stem cell therapy for the treatment of acute myocardial infarction and HF. In total, 11 studies evaluated the efficacy of adult MSCs in acute myocardial infarction, while 12 studies evaluated their efficacy in ischemic HF. Post-treatment; there was a significant improvement in LVEF but no significant differences in mortality between groups (Lalu et al. 2018). However, upon further subgroup analysis, no significant LVEF improvements were observed in the treatment of HF, but rather only for the treatment of AMI. Positive results were observed in other clinical outcomes of HF, as there were significant improvements in quality of life and the 6-min walk test. Evidence suggests that MSC therapy seems to be safe, as there was no association between treatment and acute adverse outcomes for patients. An important limitation to consider is that MSCs are expected to lose some form of efficacy due to aging, warranting donations from healthy donors rather than obtaining autologous cells from older individuals who may have additional comorbidities (Tehzeeb et al. 2019). Cardiopoietic stem cells are more specialized

cells derived from a pure MSC population in the bone marrow. Although these are more developed than true MSCs, they have been of interest in HF studies. The C-CURE trial is one of the first using cardiopoietic cells in the treatment of HF. Findings demonstrated an increased LVEF, improved quality of life, and a lower left ESV after 2 years (Bartunek et al. 2013). The findings of the C-CURE trial catalyzed larger studies to take place such as the CHART-1 trial, which shared similar results as the latter. Both the C-CURE and CHART-1 trial indicate that stem cell therapy is safe and has potential to provide long-lasting benefits on cardiac function in those affected by HF (Bartunek et al. 2017). Larger randomized controlled trials, along with a comprehensive assessment of the impact of MSCs on cardiac function, would further establish a conclusive risk-benefit ratio for MSCs.

Skeletal Myoblasts

Early preclinical trials showed promise as skeletal myoblasts appeared to have the capabilities to differentiate into cardiomyocytes and improve cardiac function in animal models (Taylor et al. 1998; Chiu et al. 1995). Other preclinical studies were not able to successfully transdifferentiate skeletal myoblasts into cardiomyocytes after grafting, contradicting earlier evidence (Reinecke et al. 2002). Regardless, skeletal myoblasts were quickly rushed into clinical trials, and the results were disappointing. In the MAGIC trial, the intramyocardial injection of skeletal myoblasts did not improve LVEF and failed to improve regional and global heart function. In addition, patients receiving the skeletal myoblasts had a significantly greater risk of arrhythmias and embolic events compared to the placebo (Menasché et al. 2008). Similarly, the MARVEL trial did not demonstrate improvements in left ventricular function, although some moderate improvements in the distance for the 6-min walk test were noted (Povsic et al. 2011). The MARVEL trial also revealed that intramyocardial injection of skeletal myoblasts posed an increased risk of developing ventricular tachycardia. Since these landmark trials have come out, researchers have transitioned away from using skeletal myoblasts in hopes of finding a safer, more effective alternative cell type.

Pluripotent Stem Cells

Pluripotent stem cells include both ESCs and iPSCs and, by definition, are cells that can form all three germ layers of the embryo (Hackett and Fortier 2011). Although there are subtle differences in potency between the two cell types, the major distinction between the two comes from their difference in origin. Nonetheless, pluripotent stem cells (PSCs) have a unique advantage of being able to be differentiated in a tightly controlled, stepwise fashion. This allows researchers to create lineage-specific progenitors such as CPCs at their preferred level of differentiation (Menasché 2018).

Embryonic Stem Cells

There have been relatively few trials investigating the safety and efficacy of human ESCs in preclinical and human models. Of interest, two preclinical trials in non-human primates involved intramyocardial delivery of human ESC-derived

cardiomyocytes post-ischemia/reperfusion injury (Liu et al. 2018; Chong et al. 2014). In these studies, human ESCs derived cardiomyocytes were administered 2- and 4-weeks post-MI into immunocompromised Macaque monkeys. These studies produced some positive results: as hearts exhibited significant remuscularization within the infarcted area, ESC-grafts demonstrated successful reperfusion by the host vasculature, and engrafted cells demonstrated electromechanical coupling to host myocytes. There was also no sign of immune rejection or teratoma formation. However, there was no significant improvement in LVEF and non-fatal ventricular arrhythmias were seen in all monkeys (Liu et al. 2018; Chong et al. 2014) These findings were reproduced in a similar preclinical experiment administering human ESC derived cardiomyocytes into a post-MI porcine model (Romagnuolo et al. 2019). Together, these studies demonstrated the feasibility of producing and using human ESC derived cardiomyocytes on a clinical scale and opened the door for phase 1 clinical trials in humans.

The first human trial using human ESC derived CPCs to treat HF was completed and illustrated some promising preliminary results (Menasché et al. 2018). This trial incorporated 6 patients with left ventricular dysfunction (ejection fraction <35%) and a history of myocardial infarction. A fibrin patch embedded with human ESC derived CPCs was implanted epicardially during a coronary artery bypass procedure. At the endpoint of 1 year, there were no signs of teratoma formation or arrhythmias present in any of the patients. Interestingly, the four patients who were assessed at the 1-year follow up had functional and symptomatic improvements. Although statistically insignificant, they showed a modest increase in LVEF, a decrease in left ventricular volume, and a statistically significant improvement in heart wall motion within the cell treated segments. Patients also showed symptomatic improvement quantified by a decrease in the New York Heart Association Class and an increased 6-min walk test. Although these results regarding efficacy do appear quite exciting, they should be interpreted with caution as it was an extremely small sample size and there are various confounding variables involved, including the concomitant coronary artery bypass grafting. However, the principal discovery of this trial was successful in showing that human ESC derived CPCs can be produced on a clinical scale and show no major signs of adverse effects after implantation. This trial does display the potential for human ESCs to be used in the treatment of HF, and further clinical trials are warranted to investigate the full extent of their clinical usefulness.

Induced Pluripotent Stem Cells

Since the major discovery of Takahashi and Yamanaka in 2006, there has been great interest in the therapeutic potential of iPSCs. The first human clinical trial involving iPSCs occurred in 2014 and was aimed at treating age-related macular degeneration (Mandai et al. 2017). Although the trial didn't produce positive results in terms of clinical improvement, it demonstrated the feasibility of human iPSCs being produced on a clinical scale and administered to humans without major safety concerns. From there, iPSCs began being investigated for various diseases, including Parkinson's disease, immunotherapy for cancer, and now

heart disease (Bragança et al., 2019). Several pre-clinical studies have validated that iPSCs could play an important role in cardiac repair. Ye et al. (2014) demonstrated that intramyocardial administration of a fibrin patch embedded with human iPSC derived cardiomyocytes among other cells and growth factors produced a significant improvement in left ventricular function and decreased infarct size in a post-MI porcine model. In a recent study, extracellular vesicles secreted by murine iPSCs were shown to cause a significant improvement in left ventricular function and a decrease in infarct mass in a post-MI mouse model (Adamiak et al. 2018).

There are currently two clinical trials that have been approved for utilizing iPSCs in the treatment of chronic cardiomyopathy in humans. The world's first clinical trial was approved in Japan in 2018 and aims to administer a patch of human reprogrammed iPSC cardiomyocytes into the damaged myocardium (Cyranoski 2018). Details about the trial are scarce, but three initial patients with chronic ischemic cardiomyopathy have been treated and the clinical trial aims to involve 10 patients over 3 years. Follow up will occur at 1-year post-implantation and the primary endpoints investigated will be safety and efficacy. The second clinical trial (HEAL-CHF) is an open-label taking place in China. Five patients with HF will be treated with intramyocardial delivery of allogeneic human iPSC-derived cardiomyocytes and assessed for safety and efficacy. There are currently no published results from either trial, although these should be expected within the next year.

One of the major barriers that arose during preclinical trials is that cardiomyocytes derived from PSCs have an immature phenotype compared to human adult cardiomyocytes. The ideal transplant would involve cardiomyocytes integrating into the host myocardium, secreting factors to re-vascularize necrotic tissue, electromechanically coupling with the resident cardiomyocytes, and beating in synchrony (Gerbin and Murry 2015). Unfortunately, human PSC derived cardiomyocytes are functionally immature in terms of their sarcomere organization, calcium handling properties, and metabolism compared to adult cardiomyocytes (Mazzola and Di Pasquale 2020). This limits their ability to efficiently integrate with host cardiomyocytes and is believed to be the reason that ventricular arrhythmias can arise (Vagnozzi et al. 2018). The problem may not be with the potency of the cells themselves, but rather the differentiation techniques that are currently used to create the cardiomyocytes. Strategies that enhance the differentiation of PSC-derived cardiomyocytes include the use of bioengineered scaffolds, chemical factors, mechanical loading, and electrical stimulation (Ibid.). Since then, we have seen the emergence of the very first human clinical trials in both ESCs and iPSCs in the treatment of HF and more are likely to come (Menasché et al. 2018; Cyranoski 2018). Although data is still quite limited, initial results regarding safety are quite promising, suggesting that the challenges of cell integration surrounding the immature cardiomyocyte phenotype may not be as severe in humans. Future studies should shift toward confirming safety in larger cohorts and optimizing the efficacy of PSCs. A summary of some of the landmark clinical trial has been included in Table 1.

Table 1 Summary of landmark clinical trials

Clinical trial	Cell type	Study design	Efficacy	Safety	Conclusions
1. SCIPIO (2011)	Kit+ cardiac	Phase I randomized open label 23 patients total Intracoronary injection	Decrease in infarct size and increase in LVEF	Satisfactory safety profile	Improvements in ventricular function "Expression of concern" from *The Lancet,* 2014
2. CONCERT-HF (2015–present)	Kit+ cardiac and bone marrow-derived MSCs	Phase II randomized, placebo-controlled study 144 patients total Transendocardial injection	Awaiting results	Awaiting results	Awaiting results
3. TAC-HFT-II (estimated start date March 1, 2025)	Kit+ cardiac and bone-marrow derived MSCs	Phase I and II randomized, placebo-controlled trial 55 patients total	Awaiting results (2032 estimated completion date)	Awaiting results	Awaiting results
1. ALLSTAR (2017)	Cardiosphere-derived	Phase II randomized double-blind placebo-controlled 134 patients total Intracoronary injection	Study was stopped prematurely	Study was stopped prematurely	Study was stopped prematurely
2. CADUCEUS (2014)	Autologous cardiosphere-derived	Phase I randomized open label 25 patients total Intracoronary injection	Reduction in infarct size and improvements in contractility	Satisfactory safety profile	Structural improvements but no improvements in LVEF
1. FOCUS CCTRN (2012)	Autologous bone marrow-derived mononuclear	Phase II randomized double-blind placebo-controlled trial 92 patients total Transendocardial injection	No significant improvements in LVEF noted	No in-hospital events besides one aortic dissection No other safety concerns noted	Transendocardial injection of bone marrow cells versus placebo did not improve LV end systolic volume, maximal oxygen consumption, or perfusion defects

(continued)

Table 1 (continued)

Clinical trial	Cell type	Study design	Efficacy	Safety	Conclusions
2. TAC-HFT-I (2009–2013)	Autologous bone marrow-derived mononuclear and MSCs	Phase I and II randomized blinded placebo-controlled trial 65 patients total Transendocardial injection of MSCs and placebo, and BMCs with placebo	Improvements in the MLHF score, 6-min walk distance, infarct size, and regional function with MSCs only	Transendocardial injection was not associated with an increased risk of side effects	Ejection fraction and left ventricular chamber volume did not change. Larger studies are warranted to provide definitive evidence about safety and assess efficacy
3. STAR-heart (2003–2005)	Autologous bone marrow-derived mononuclear	Prospective study 191 patients total Intracoronary injection	Improvements in NYHA functional class, decreased LV preload, end-systolic volume, wall stress, arrhythmias, and smaller area of infarction	No side effects were documented	Intracoronary injection of autologous bone marrow cells decrease long-term mortality and improve ventricular function
1. POSEIDON (2010–2011)	Mesenchymal	Phase I/II randomized comparison 30 patients total T.E. injection	Both allogeneic and autologous MSCs reduced infarct size, adverse cardiac remodeling and LV function	Satisfactory safety profile	Patients treated with both allogeneic and autologous MSCs had functional and structural improvements over time
2. MSC-HF (2015)	Mesenchymal	Phase I/II randomized double-blind placebo-controlled trial 60 patients total Intramyocardial	Improvements in LVEF, stroke volume, and myocardial mass	Satisfactory safety profile	Autologous MSCs improved myocardial function and myocardial mass in heart failure patients

3. CHART-1 (2017)	Cardiopoietic	Phase III prospective, randomized double blind placebo-controlled trial 271 patients total I.M. injection	Significant benefits with patients with severely dilated ventricles	Satisfactory safety profile	Neutral outcome with no significant difference between all-cause mortality, rehospitalization for MI, heart failure, and stroke at 12 months between groups
4. DREAM-HF (2014)	Allogeneic mesenchymal precursor cells	Phase III randomized double-blind placebo-controlled trial 566 patients total T.E. injection	Awaiting results	Awaiting results	Study is expected to be completed on May 29, 2020
1. MAGIC (2008)	Skeletal myoblasts	Randomized, placebo-controlled double-blind study in 120 patients Conducted in 21 hospitals in France, Italy, Germany, Belgium, UK, I.M. injection	No significant improvements in LVEF noted	Risk of arrhythmias	Unsafe and ineffective source of stem cells
2. MARVEL (2011)	Skeletal myoblasts	Randomized placebo-controlled trial 23 patients total I.M. injection	No improvements in LV function Moderate improvements in 6-min walk distance	Increased risk of ventricular tachycardia	Mere investigations are warranted to determine improvements in functional capacity
1. ESCORT (2018)	Embryonic	Open label interventional trial in 6 patients Epicardial delivery of human ESC derived cardiomyocytes embedded in a fibrin patch Primary endpoint at 1 year	Decrease in New York Heart Association functional class Modest decrease in LV volumes and an increase in LVEF Improved systolic wall segment motion	No signs of teratoma formation or arrhythmias at 1 year – 1 patient died postoperatively from unrelated comorbidities	Demonstrates feasibility of clinical grade human ESCs Demonstrated positive safety profile Moderate improvements in cardiac function and symptomatic improvement

(continued)

Table 1 (continued)

Clinical trial	Cell type	Study design	Efficacy	Safety	Conclusions
1. HEAL-CHF (ongoing)	Induced pluripotent	Open label interventional trial in 5 patients Direct epicardial injection of allogeneic iPSC derived cardiomyocytes	Awaiting results (estimated December 2020 completion)	Awaiting results	Awaiting results
2. Induced pluripotent trial Japan (initiated May 2018)	Induced pluripotent	Scarce details 7–10 patients total Administration of human iPSC derived cardiomyocytes	Awaiting results (2021 completed date)	Awaiting results	Awaiting results

Insights into Ccell-Based Therapy Approaches

What Is the Proposed Mechanism of Action?

In general, regulatory bodies such as the FDA and Health Canada require a clear and concise mechanism of action for most drugs and molecular therapies to be approved (Overington et al. 2006). However, the mechanism of action for stem cell therapy in the treatment of cardiovascular disease appears quite complex. It has still not yet been fully elucidated, creating a barrier for clinical trials. Initially, it was believed that transplanted stem cells differentiated into functional cardiomyocytes and replaced the damaged tissue within the myocardium. This was supported by evidence showing that transplanted BMDSCs engrafted into damaged myocardium of mice produced a substantial amount of proliferating myocytes and vasculature within the infarcted tissue 9 days post-injection (Orlic et al. 2001). There is also evidence suggesting the fusion of the transplanted stem cells with the resident cardiomyocytes (Nygren et al. 2004). However, in 2004 a landmark study showed that retention of transplanted hematopoietic stem cells into the recipients' hearts was extremely low, and there was a lack of significant differentiation into cardiomyocytes (Murry et al. 2004). This provided the first clue that direct cardiomyocyte replacement may not be the primary mechanism of repair. Since then, a study revealed that the administration of cultured medium from adult MSCs alone produced the same beneficial effects as MSCs in vitro and in vivo (Gnecchi et al. 2005). Although there has been convincing evidence that engraftment of transplanted stem cells does improve cardiac function, the small number of cells retained couldn't have solely produced the clinical benefits seen (Hatzistergos et al. 2010). The leading hypothesis then transitioned toward transplanted stem cells providing cellular support via the secretion of paracrine factors. Although the mechanism of action is likely multidimensional, it is now widely accepted that the vast majority of physiologic effects come from paracrine signaling (Bruyneel et al. 2016). However, the mechanism likely also varies with each cell type and the route of administration.

Paracrine Signaling

In the context of cardiac regeneration, paracrine signaling involves the release of various effector substances that directly cause cardioprotective effects or active endogenous regeneration mechanisms within the heart. These include various chemokines, cytokines, growth factors, and extracellular vesicles called exosomes that contain proteins and RNA (Bruyneel et al. 2016). Stem cells are believed to release these substances and alter the existing environment within the region they are transplanted in (Tang et al. 2018). Although the paracrine factors vary between stem cell types, it is believed that these factors activate endogenous repair mechanisms including neovascularization, inhibiting apoptosis, mitigating inflammation, and many others (Oikonomopoulos et al. 2018). For example, adult stem cells, including MSCs or BMDSCs, have been shown to secrete vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (Gnecchi et al. 2008). IGF-1 is

believed to act as a cardio-protective factor to inhibit cardiomyocyte apoptosis via activation of the Akt pathway (Nagaya et al. 2005). VEGF, among other growth factors, is believed to be the key to stimulating the formation of new blood vessels (Guo et al. 2017). A comprehensive list of all the secreted paracrine factors and their mechanisms has yet to be determined, making it difficult to identify which combination of factors is most effective at promoting cellular regeneration. Regardless, transplanted stem cells are believed to release a large number of paracrine factors, which activate a network of pro-survival cascades, leading to an overall cardioprotective effect (Bruyneel et al. 2016).

Direct Regeneration

Although the paracrine signaling theory predominates most of the literature in the CSC field, recent advancements in pluripotent stem cell medicine have somewhat reignited the idea that direct engraftment may contribute to heart regeneration (Liu et al. 2018; Chong et al. 2014; Ye et al. 2014; Adamiak et al. 2018). Both ESCs and iPSCs have previously been shown to be successfully differentiated into cardiomyocytes (Kehat et al. 2001; Batalov and Feinberg 2015). However, only recently has there been evidence that PSC derived cardiomyocytes can successfully integrate into the host heart, form mature cardiac grafts and electromechanically couple with the host myocytes (Liu et al. 2018). Although this was in a non-human primate model, it still alludes to the fact that long term cellular integration of human PSC derived cells into a host heart is plausible. As transplantation and differentiation techniques improve, we may see an increase in the long-term cellular engraftment rates into the host heart and therefore, an increase in the overall efficacy of stem cell transplantation that is considered multifactorial as is depicted in Fig. 1.

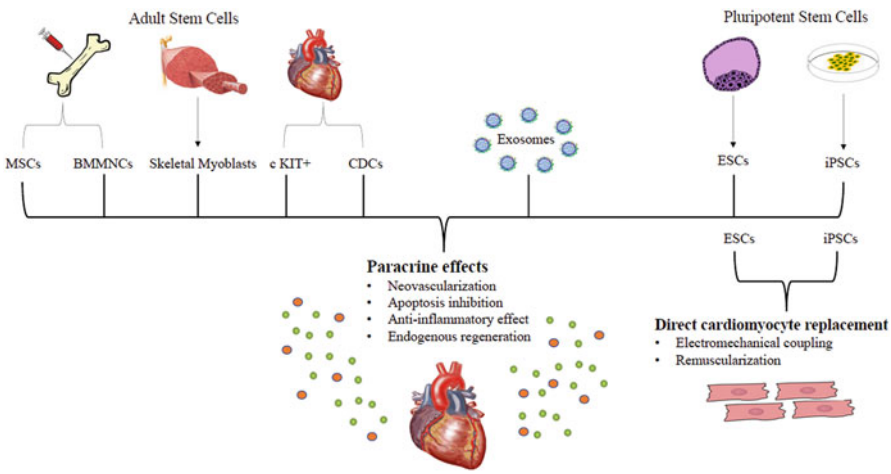

Fig. 1 Proposed mechanism of action for stem cells in the treatment of heart failure. This diagram shows various adult stem cell and pluripotent cell types and their contribution to cardiac regeneration

Optimizing Cardiac Stem Cell Therapy

In treating HF, successful therapy will depend on multiple factors: the type of stem cells, the route of administration, the timing of the therapy, and the characteristics of the patient.

What Is the Optimal Cell Type?

So far, multiple cell types have been proposed as the next generation of treatment for HF. These include various classes of adult CSCs, MSCs, non-selective bone marrow-derived stem cells, skeletal myoblasts, iPSCs, and ESCs. Unfortunately, determining the optimal cell type for the treatment of HF is not a straightforward endeavor. Many practical factors regarding the delivery of cells need to be considered before definitively crowning a superior cell type (Faiella and Atoui 2016). In addition to determining clinical efficacy, careful consideration of ethical concerns, simplicity of treatment, and patient preferences will also influence which cell type will predominate future treatment options (Raval et al. 2008). Determining the optimal cell type requires a comprehensive understanding of the mechanism of action of cell-based therapies. Currently, the proposed mechanism of myocardial repair involves either direct replacement of functional, contractile cardiomyocytes, or paracrine signaling to activate endogenous repair mechanisms within the heart (Nakamura and Murry 2019), although these events are not mutually exclusive. Thus, cellular therapies aim to achieve either one or both of goals, depending on the type of cell.

Skeletal myoblasts were one of the very first cells to be tested in the treatment of HF (Garbern and Lee 2013). There are many features that make skeletal myoblasts a viable option for cardiac repair such as their resistance to ischemia, high proliferative potential, and myogenic capabilities (Durrani et al. 2010). However, a significant challenge surrounds skeletal myoblasts, as they appear to remain electromechanically independent from the host myocardium (Ferreira-Cornwell et al. 2002). This was exhibited in the landmark MAGIC trial, where patients receiving skeletal myoblasts had a higher rate of arrhythmic events than those receiving the placebo. In addition, the skeletal myoblast administration did not improve heart function (Menasché et al. 2008). Since then, the interest in using skeletal myoblasts in cardiac cellular therapeutics has diminished, as more attractive cell candidates have emerged (Rikhtegar et al. 2019).

BMDSCs were also among the first cell types to be tested, with BMMNCs and MSCs being the most heavily investigated. Initially, it was shown that the infusion of BMDSCs could regenerate the myocardium (Beltrami et al. 2001). However, as time progressed, the primary hypothesis shifted toward paracrine signaling, as there is little evidence of long-term engraftment in the heart (Turner et al. 2020). After a decade of research, a trial sequential analysis reported mixed results. It demonstrated that the administration of BMMNCs caused a reduction in all-cause mortality and hospitalization from HF. The analysis also showed cell therapy did not improve LVEF by a value of more than 4% (Fisher et al. 2016b). In contrast, there have been mainly positive results published about MSCs. Three separate studies have demonstrated a robust safety profile surrounding MSCs and may have a clinical impact on LVEF, quality of life and cardiac remodeling (Hare et al. 2012; Rikhtegar et al. 2019; Mathiasen et al. 2020). Interestingly, the TAC-HFT trial compared BMMNCs versus

MSCs in a head-to-head randomized phase 2 trial. They demonstrated that bone marrow-derived MSCs produced significant improvements in quality of life, 6-minute walk test, and infarct size, while BMMNCs did not (Heldman et al. 2014). These findings suggest that MSCs may have superior capabilities in producing anti-fibrotic effects than BMMNCs (Banerjee et al. 2018). However, larger comparative studies are needed before we make definitive statements on efficacy.

C-kit+ CSCs and cardiosphere derived cells have been the two most heavily studied CSCs to date. Like MSCs, CPCs have minimal long-term engraftment, suggesting that their primary mechanism of action is through paracrine signaling (Tang et al. 2016). Landmark studies such as SCIPIO and CADUCEUS have demonstrated a positive safety profile for both C-Kit+ and CDCs. They have also shown varying degrees of cardiac recovery, with one study demonstrating structural improvements, one study exhibiting improvements in ventricular function, and both showing a decrease in infarct size (Chugh et al. 2012). However, it is difficult to conclude which cell type is superior to the other because there has yet to be a comparative trial completed. Despite the lack of a comparative trial, the initial clinical results surrounding CPCs are promising, and we remain optimistic that ongoing studies such as the CONCERT trial will produce positive results.

ESCs and iPSCs hold the highest level of potency, and therefore their primary advantage is the ability to effectively differentiate into functional cardiomyocytes or cardiovascular progenitors. In comparison to other cell types, PSCs also appear to hold a distinct advantage: the ability to integrate and electromechanically couple to the host myocardium (Liu et al. 2018). Theoretically, this means that PSCs can generate beneficial effects via both paracrine signaling and direct cardiac replacement. Perhaps this could be the underlying reason that ESCs see early success in preliminary clinical trials (Menasché et al. 2018). Nevertheless, there are still some safety concerns that arise when discussing PSCs, including ventricular arrhythmias, teratoma formation, and immune rejection (Gerbin and Murry 2015). We will need to see higher-powered clinical trials demonstrating a positive safety before we can make any definitive statements on the potential of pluripotent stem cells. Still, we believe that human ESCs and iPSCs are truly one of the top candidates to represent the next generation of cellular therapeutics.

Alternatively, there may not be one optimal cell type but rather an optimal combination of stem cells. This strategy could take advantage of the diverging mechanisms of action between cell types and synergistically (Turner et al. 2020). This theory has been validated in numerous preclinical studies, where the combination of CSCs and MSCs provided a significant improvement in cardiac function compared to either cell type alone (Natsumeda et al. 2017; Karantalis et al. 2015). Researchers applied this knowledge to create the first clinical trial comparing different types of stem cells in the treatment of chronic cardiomyopathy (Bolli et al. 2018). The CONCERT study is an ongoing phase 2 clinical trial assessing the head-to-head comparison of c-Kit+ CSCs alone, MSCs alone, and the combination of the two. Additionally, there was a recently published study demonstrating that concomitant administration of human iPSC derived cardiomyocytes and human MSCs loaded on a patch produced a synergistic effect on cardiac repair (Park et al. 2019).

Ultimately, we need randomized controlled trials comparing the efficacy of stem cell types before we can make any definitive recommendations on what the superior

cell type is. There is currently a lack of robust evidence assessing the superiority of one stem cell type versus another via human clinical trials. To date, the only published study directly comparing cell types in the treatment of HF has been the TAC-HFT trial. We will likely see an increase in comparative studies such as the CONCERT trial in the upcoming years as more evidence surrounding the optimal administration of stem cells is produced.

What Is the Optimal Method of Delivery?

One of the most important factors in successful cell therapy is the route of administration to the host (Turner et al. 2020). Though the idea may seem simple, there is a need for a method of delivery that will deliver the cells that we want into the correct area, which the cells delivered will engraft effectively while being able to function with the heart's natural rhythm without interference. Currently, there is no consensus on the most effective route, yet most studies suggest that the infusion, injection, or tissue-based implantation of cells can present therapeutic benefits to injured myocardium (Eschenhagen et al. 2017). Multiple routes have been studied preclinically: intravenous, intracoronary, intramyocardial, percutaneous transendocardial, transendocardial, retrograde intracoronary sinus, open surgical epicardial injection and scaffolding (Nakamura and Murry 2019; Bruyneel et al. 2016; Sudulaguntla et al. 2017) (Fig. 2).

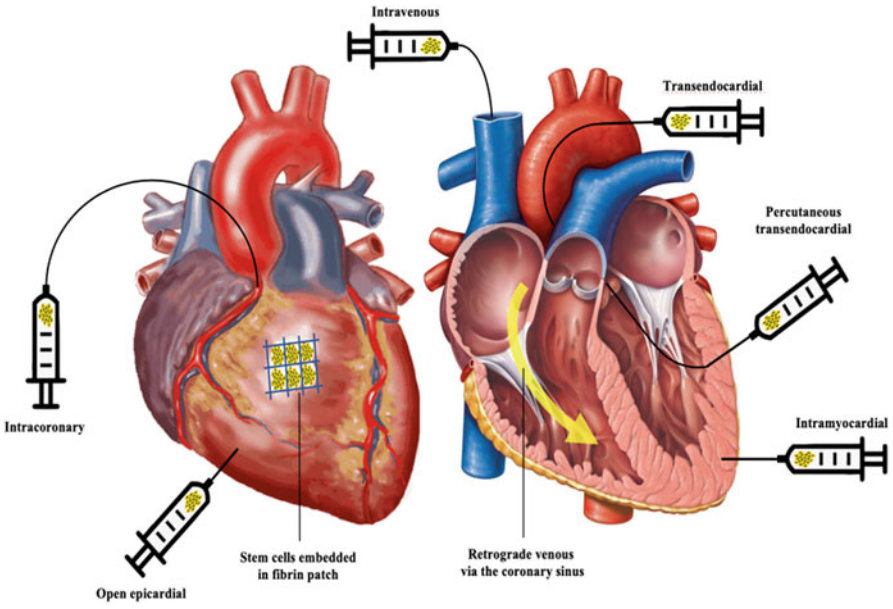

Fig. 2 Schematic depicting the various routes of delivery of stem cells. On the left, we see the intracoronary method, epicardial injection and cells embedded in a scaffold made of fibrin. On the right, we see the intravenous method, the retrograde venous route via the coronary sinus, the transendocardial method via the aorta, the percutaneous transendocardial method, and intramyocardial injection

The intravenous approach is attractive, as it is a straightforward procedure. It is currently the least invasive and has been demonstrated to have a positive safety profile (Menasché 2018). Intravenous injection has been found to attract cells to the site of injury, although there are concerns of poor cell implantation and retention (Turner et al. 2020). Intracoronary infusion of stem cells is the most common route of administration observed in studies and causes minimal inflammation. It is associated with some paracrine effects but, unfortunately, minimal cell retention and rapid washout in humans, resulting in inefficient re-muscularization (Nakamura and Murry 2019). The STAR-heart Study showed several benefits on cardiac function and minimal adverse effects with intracoronary infusion (Tehzeeb et al. 2019). Large doses of stem cells cannot be delivered by intracoronary infusion as these can obstruct coronary arteries and cause ischemia, leading to myocardial cell death (Nakamura and Murry 2019). Although there are potential risks involved, intracoronary administration of stem cells is one of the safer techniques of delivery (Tehzeeb et al. 2019). Since both intracoronary and IV administration do not confer high rates of long-term engraftment, stem cells that produce a strong paracrine effect should be selected. This means that adult stem cells such as MSCs and CSCs would be better candidates than PSCs in conjunction with this route of administration.

Intramyocardial injection of stem cells has a superior engraftment capability but has a greater arrhythmic potential, is more invasive, and increases the risk of perforating the myocardium (Nakamura and Murry 2019). The transendocardial route is minimally invasive, and the POSEIDON trial demonstrated a positive safety profile (Tehzeeb et al. 2019). Though this method carries a small risk of perforation and arrhythmias, cell retention is more successful versus other methods (Turner et al. 2020). It also permits the visualization of the target area, allowing greater accuracy injecting the target site. Currently, there are a limited number of studies and insufficient data on the safety profile of retrograde intracoronary sinus injections (Gathier et al. 2018). A small randomized control study with 20 patients with ischemic HF had autologous stem cells administered to the sub-epicardial regions of the heart plus CABG (Tehzeeb et al. 2019). Results showed a significant increase in LVEF and no adverse effects were observed.

Over the last few years, bioengineering has crept into the field of regenerative medicine. This involves culturing and implanting stem cells in 3D environments to improve both cell differentiation and survivability. One of the current goals is to create a scaffold that mimics the microenvironment of the heart and can be grafted with your cell type of choice into the heart (Mazzola and Di Pasquale 2020). The first application of cardiac patches was in a clinical trial instrumenting a fibrin scaffold embedded with human ESC-derived CPCs (Faiella and Atoui 2016). This method has been validated in preclinical models and appears to yield higher rates of cell retention (Park et al. 2019). In addition to fibrin, other biomaterials are used in scaffolding. Notably, natural materials like collagen, hyaluronic acid, and alginate, as well as a large variety of synthetic polymers have shown great variability in advantages and disadvantages (Mazzola and Di Pasquale 2020). Although the optimal combination of stem cell types and scaffolding materials has yet to be

confirmed, PSCs seem to be one of the better candidates for engineering functional heart tissue (Oikonomopoulos et al. 2018). This is because PSCs have a greater potential to integrate into the host myocardium than adult stem cells (Liu et al. 2018). Two main types of tissue engineering are currently being investigated: hydrogel-based and cell sheet engineering (Oikonomopoulos et al. 2018). Very few studies have directly compared therapeutic differences between routes of administration of stem cells. Clinical studies have yet to identify the optimal route of administration, and the most efficacious method is likely cell-type dependent (Turner et al. 2020).

What Is the Appropriate Timing of Administration?

This variable is also of great importance, as administering cells in the acute (less than 30 days), subacute (between 30 days and 1 year) or chronic phase (more than 1 year) post-MI is expected to work by different mechanisms and requires different cell properties (Nakamura and Murry 2019). Administration of cells in the acute phase aims to control inflammation and cardiomyocyte apoptosis while increasing vascularization. Conversely, in the subacute or chronic phase, therapy aims to augment repair of the myocardium and replace lost tissues. Multiple studies suggest that the administration of stem cells in 2–8 days post-MI is the most favorable window to modulate injury response and potentiate repair mechanisms. The exact dosing has yet to be confirmed and remains under investigation (Ibid.).

Patient Characteristics and Sourcing

Currently, patient characteristics that may improve or impede the success of cell therapy have not yet been studied systematically. Poor patient parameters such as diabetes, smoking, obesity, and older age can impair both autologous cell potency and the host's receptivity to stem cell treatments (Nakamura and Murry 2019). Studies are also comparing cells from autologous and allogeneic sources. Autologous cells have the benefit of immunocompatibility to the host, but their potency may be negatively impacted by poor patient parameters. In addition, autologous therapy is currently impractical for replacing billions of cells and for diseases that require treatment within a few weeks, such as a MI. Allogeneic cell therapy allows for increased availability, scalability, and quality-controlled product development (Ibid.). To receive allogeneic cardiomyocyte transplantation, patients are required to be chronically immunosuppressed, to which the degree is unknown at the moment (Pidala et al. 2011).

What Are Some Challenges that Remain?

Stem cells show exciting potential for the future, but several complexities must be addressed before widespread applications in the treatment of HF becomes feasible. We've seen varying levels of success for different cell types and the results have yet to be uniform and consistent in studies. Of the many challenges that remain, tissue integration is a key component to master, in order to produce myocardial tissue that acts as a functional syncytium. The ideal stem cell will have to effectively engraft into the heart to repair the damaged area, create new functional cardiac tissue and contract with the heart's natural rhythm in a coordinated fashion. So far, it seems like

iPSCs and ESCs-derived CPCs have the greatest potential to integrate mechanically and electrically into the myocardium (Liu et al. 2018). A direct comparison of the advantages and disadvantages of stem cells has been summarized in Table 2.

Table 2 Advantages and disadvantages of various stem cell types

Cell type	Advantages	Disadvantages
Cardiac stem cells	Autologous transplantation → decreased risk of immune rejection Low risk of carcinogenicity Moderate level of potency → can differentiate into various cardiac specific cell types	Difficult to isolate from myocardium → requires invasive biopsy Lack of consistency provided from clinical trials
Nonselective bone marrow-derived stem cells	Improves structural recovery of the heart and decreases infarct size Positive safety profile in multiple human clinical trials Autologous or allogeneic transplantation	Low quality evidence on efficacy in humans Minimal functional improvements on cardiac function Requires invasive biopsy
Mesenchymal stem cells	Positive safety profile demonstrated in multiple human clinical trials Autologous transplantation → lower risk of immune rejection Positive safety profile for allogeneic transplantation Exhibit strong paracrine effects	Difficult to isolate from bone marrow, although umbilical MSCs from Wharton's Jelly appear more accessible Quality of autologous cells may be affected by aging
Embryonic stem cells	High level of potency and controlled differentiation Well established cell lines Can produce cardiomyocyte progenitors on a clinical scale Minimal genetic manipulation of cells Indefinite self-renewal of cells	Has to be allogeneic → increased risk of immune rejection Ethical concerns about destruction of potential human life Risk of teratoma formation Risk of ventricular arrhythmias → in preclinical annual models Currently only 1 human clinical trial completed
Induced pluripotent stem cells	High level of potency and controlled differentiation Can be autologous transplantation → increased risk of immune rejection Less ethical concerns as they are derived from somatic cells Easily accessible source of cells	Increased risk of teratoma formation compared to ES cells due to use of viral vectors Variability in completeness of reprogramming No published human clinical trials completed
Skeletal myoblasts	Autologous transplantation → decreased risk of immune rejection Low ethical concerns Low risk of carcinogenicity	Low differentiation potential Unsuccessful in differentiating into functional cardiomyocytes after grafting Risk of ventricular arrhythmias → in human clinical trials Risk of embolism → shown in human clinical trials

PSCs also carry the potential for transplanted cells to form teratomas (Thomson et al. 1998; Takahashi et al. 2007). Thus, it is important to precisely differentiate cells pre-transplant to avoid the development of malignancies. However, the directed differentiation of PSCs is a challenge. Currently, techniques to completely differentiate PSCs into a fully functional, mature, cardiomyocyte phenotype have proven difficult. PSC derived cardiomyocytes appear functionally immature in terms of their sarcomere organization, calcium handling properties, and metabolism compared to adult cardiomyocytes (Mazzola and Di Pasquale 2020). This is believed to be the reason that ventricular arrhythmias were arising during preclinical trials (Vagnozzi et al. 2018).

Other challenges include the matching between patient and donor. A close match between parties is essential, as it reduces the risk of cell rejection and the need for lifelong immunosuppressants. This challenge may be mitigated by developing patient-specific iPS cell lines that could potentially avoid rejection and the need for immunosuppression (International Society for Stem Cell Research n.d.). Autologous cells are also an option, but the capacity of producing clinical-grade autologous cells during an acute time frame is currently not realistic. Additionally, potency may be impacted by poor patient parameters as previously explained. Lastly, we need an abundant source of stem cells available with hopes of treating many patients across the world. We need to identify, isolate, and grow the correct type of stem cell to have the therapy available and avoid shortages. ESCs and iPSCs are great candidates as they can be grown indefinitely in the laboratory, but this is a complex process that is tightly regulated (International Society for Stem Cell Research n.d.).

What Are Some Ethical Considerations in Using Stem Cells?

Stem cell research offers a novel solution for understanding and treating a variety of human diseases, including HF (National Research Council 2002). However, this research generates ethical, religious, and political controversies.

The most obvious controversy surrounding stem cells revolves around the morality of ESCs. Human ESCs are derived from the inner cell mass of the blastocyst from day 5–7 before implantation. This process involves the destruction of the human embryo and, thus, the potential for human life. This begs the question: When does human life begin? Some believe that human life begins as soon as fertilization occurs, and a zygote is formed. This view is often known as "pro-life" and believes human embryos have the same rights and interests of all humans. From this "pro-life" perspective, the destruction of the blastocyst pre-implantation is equal to murder (Lo and Parham 2009). Two solutions have been implemented by the Canadian Institutes of Health Research, which partially circumvent the morality issues associated with ESCs (Government of Canada and Interagency Advisory Panel on Research Ethics 2018). The first guideline strictly inhibits the creation of embryos for the sole purpose of research. The second guideline stipulates that the embryo must have been created for reproductive purposes. On the other hand, many others believe the embryo has a different moral status and is not considered a "person" until later on in development past fertilization. The Canadian Institutes of Health Research developed guidelines that attempt to respect the diversity of opinion

on the subject. Current guidelines state that "research involving embryos will take place only during the first 14 days after their formation by a combination of the gametes, excluding any time during which embryonic development has been suspended" (Ibid., p. 174). The final question comes down to: Is it morally acceptable to destroy the potential for human life to research new therapeutics to treat diseases? Since individuals will all have different moral beliefs on life, a definitive policy on the moral status of the embryo will likely never be globally accepted. Hence, iPSCs are increasing in popularity for research as there are fewer ethical considerations involved.

A fundamental principle in medicine is the process of containing informed and voluntary consent from patients before any medical procedure. This includes the ability to withdraw consent for a medical procedure at any time. This principle is implemented in general research by Canada's Tri-council policy, where research participants can withdraw consent at any time (Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and Social Sciences and Humanities Research Council of Canada 2014). However, in research settings involving stem cell bio-specimens, the situation becomes more complex. Ethical norms would suggest that a donor is entitled to have control over any bodily tissue that has been removed from them, including gametes or embryologic tissue. This would also include any biological health information about the donor. After all, stem cells carry the complete genome of the donor and the privacy of the donor should be protected. However, from a researchers' or clinicians' perspective, this could be severely problematic. Well established human ESC lines are often distributed to hundreds of laboratories across the world. If the donor wished to withdraw consent from the use of their biologic tissue after it had been distributed globally, it would cause significant issues and could be detrimental to the field of stem cell research (Caulfield et al. 2007). This is a realistic possibility, as the moral beliefs of donors may change with time. If the withdrawal of consent occurs within the context of a therapeutic biobank used for analogous transplantation, it would have negative repercussions for patients who are stem cell recipients. This underlines the importance of creating a Canadian policy that both respects the donors' wishes and prevents an adverse impact on stem cell research and therapeutics. Canadian consent law has yet to specifically address issues about genetic and tissue information. However, in the absence of extenuating circumstances, regulations should lean toward individual human rights and not the goals of research (World Medical Association 2000).

False Claims

Stem cell therapeutics are advancing at an unprecedented rate, and significant discoveries have been made in the last decade. The idea of treating HF with stem cells may not seem completely out of reach in the future and if proven successful, it may save millions of lives. This scientific community has displayed much enthusiasm but, in some circumstances, overstepped their boundaries and generated falsified

or embellished results. This is true in the case of Dr. Piero Anversa, who claimed that stem cells did, in fact, produce viable and functional myocardium, despite scientific consensus stating otherwise. In total, 30 publications were retracted due to disproven hypotheses and irreproducibility in laboratories (Chien et al. 2019). Patients were provided false hope and were put at risk without reliable evidence for a period of 18 years, a truly unprecedented event. This had a major impact on cardiac cell therapeutics and was disheartening for those involved in such a field. To avoid cases like this one, the International Society for Stem Cell Research (ISSCR) recognizes and confronts science hype by assuring researchers and the public that the science that is put forward is sound and treatments have proven to be safe and effective (Caulfield et al. 2016). These obligations represent a commitment to patients that stem cell research is to be undertaken ethically by following a set of standards and that treatments are provided based on proven science (Ibid.). In addition, the ISSCR provides valuable information to the public regarding facts on stem cells, treatment options, and what to ask when considering stem cell trials or therapies. Implementing a set of standards in stem cell research not only protects patients but also allows researchers to compare the outcomes of clinical trials and enables the reproducibility of such studies in clinical settings. Enthusiasm and optimism are natural components of research. However, sustained hype with lack-of supporting evidence cannot be viewed positively. For this reason, the ISSCR urges stem cell researchers to "promote accurate, balanced and responsive public representations of stem cell research" (International Society for Stem Cell Research 2016, p. 28) to ensure that risks, benefits, and uncertainties are not misrepresented. These guidelines will reduce misinformation, facilitate the provision of accurate, credible information to the public, and improve transparency in the field of stem cell therapeutics.

Conclusion

Myocardial infarction is one of the leading causes of death worldwide, and HF is an important consequence associated with the event, which can lead to catastrophic outcomes for patients. Currently, therapeutic strategies rely on treating comorbidities and improving the quality of life for patients, which is why the regenerative capabilities of stem cells have created so much promise for the field. There has recently been a drastic shift from bench to bedside studies as we have seen a large increase in human clinical trials within the last decade. However, clinical trials to date have generally produced only moderately positive or even neutral clinical outcomes in terms of efficacy. This does not imply, however, that the cells have no therapeutic value but rather, it may reflect our limited knowledge about the optimal cell type, cell dosing, method of delivery, or even the endogenous cardiac repair mechanisms. Unfortunately, the lack of significant results has generated skepticism among the scientific community, which is likely due to the tremendous expectations that have been placed on the stem cell field. However, all hope is not lost. The therapeutic use of stem cells to treat HF is still a relatively novel concept and there is a plethora of clinical trials on the horizon, which will likely provide some clarity to

the field. There are also several positive results to report from clinical trials as well. A positive safety profile has been demonstrated for MSCs, ESCs, CSCs, and BMDSCs as various preliminary clinical trials have shown the absence of significant adverse events. However, many important questions still remain. Should we be focusing on techniques that involve activating endogenous repair mechanisms within the heart? Or should we be focusing on strategies that improve the engraftment of implanted cells? What is the most effective type of cell? What is the most efficient dose and route of administration? It currently appears that the field is diverging into two: One involves using 3D bioengineered scaffolds to improve retention rates of transplanted stem cells. The other involves using no cells at all, but instead delivering exosomes suspended with proteins, DNA, microRNAs, and various other growth factors. These questions will need to get addressed before we see cellular therapeutics become a staple in the clinic. All things considered, we remain cautiously optimistic that stem cells still represent the next generation of treatment for heart failure.

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Mesenchymal Stem Cell-Probiotic Communication: Beneficial Bacteria in Preconditioning

18

Ayşegül Mendi, Büşra Aktaş, and Belma Aslım

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Abstract

Oromaxillofacial tissues are composite tissue containing teeth, bones, nerves, and blood vessels. It is a heterogeneous tissue rich in mesenchymal stem cells. So far, mesenchymal stem cells have been isolated from teeth and dental tissues, cranial bones and jaw bone marrow, and salivary glands. They have the potential to create a biological response by migrating to damaged dental tissues. However, in vitro culture conditions, reduced cell number (for periodontal ligament, gingiva tissue, buccal tissue, etc.), and unwanted differentiation mesenchymal stem cells

A. Mendi (✉)
Faculty of Dentistry Department of Basic Sciences, Gazi University, Ankara, Turkey
e-mail: aysegulmendi@gazi.edu.tr

B. Aktaş
Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Burdur Mehmet Akif Ersoy University, Burdur, Turkey
e-mail: aktas@uwalumni.com

B. Aslım
Department of Biology, Faculty of Science, Gazi University, Ankara, Turkey
e-mail: baslim@gazi.edu.tr

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during frequent passages have limited the use of such cells in clinical cell therapy. Recently, studies have focused on the precondition strategies, effect of bacteria, and their components on proliferation and differentiation of mesenchymal stem cells.

Keywords

Dental pulp · *Lactobacillus rhamnosus* · Mesenchymal stem cell · Oromaxillofacial · Precondition · Probiotics · Stem cell

Abbreviations

AP-MSCs	Apical papilla mesenchymal stem cells
CB-MSCs	Cranial bone mesenchymal stem cells
DF-MSCs	Dental follicle mesenchymal stem cells
DP*-MSCS	Dental pulp mesenchymal stem cells
EOMSCs	Epithelial and oral mucosal stem cells
G-MSCs	Gingival mesenchymal stem cells
iBM-MSCs	Iliac bone marrow mesenchymal stem cells
MSCs	Mesenchymal stem cells
OMT	Oromaxillofacial tissue
PDL-MSCs	Periodontal ligament mesenchymal stem cells

Introduction

Within the framework of International Society for Cellular Therapy (ISCT) definition, mesenchymal stem cells have the ability to adhere to plastic surfaces; self-renew; differentiate into ectoderm, endoderm, and mesoderm germ layers; and express CD73, CD90, and CD105 surface markers, while hematopoietic stem cell markers include CD11b, CD19, CD79a, CD31, CD34, CD45, and HLA-DR-negative cells (Zhou et al. 2020). Along with these features, they can be effective in their environment with cell-cell contact or in the surrounding tissues with their paracrine effects, with their secretomes of immune, hormonal, and differentiation pathways. These activities are unfolded over the last many years to encompass remarkable modulatory effects in various autoimmune and inflammatory diseases, such as graft versus host, multiple sclerosis, acute lung injury, multiple sclerosis, Crohn's disease, depression, and osteoarthritis (Zhou et al. 2020).

Embryonic stem cells (ESCs), adult somatic SCs, mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and induced pluripotent stem cells (iPSCs) have all been identified (Ding et al. 2011; Herberts et al. 2011; Egusa et al. 2012; Naji et al. 2019). Adult SCs are the ideal choice for cell-based therapy since they are free of ethical problems. MSCs are regarded as one of the most promising cell types for regenerative therapy (Naji et al. 2019). MSCs were first discovered in the bone marrow as fibroblast-like cells. At clonal density, they looked like colony-forming unit fibroblasts (CFU-Fs). Mesenchymal stem cells have been

identified from the adipose tissue, synovial fluid, uterus, pancreas, and skeletal muscle, in addition to bone marrow (Ding et al. 2011; An et al. 2015; Chen et al. 2017, 28). Bone marrow and adipose tissue have been the most commonly employed tissue sources for MSC isolation in the past. However, because the tissue collection methodology involves highly intrusive procedures, the danger of morbidity in the patient procedure area has necessitated the identification of alternative tissue sources for research (Samsonraj et al. 2017; Ducret et al. 2015; Kang et al. 2016).

After Gronthos et al. (2000) reported the identification of MSCs from dental pulp tissue, oromaxillofacial tissue mesenchymal stem cells (OMT-MSCs) were isolated and identified by different researchers (Fukumoto et al. 2003; Tatullo et al. 2015). Easy surgical access made dental pulp-derived MSCs (DP-MSCs) a promising alternative tissue source of MSCs. The easy availability of DP-MSCs compared to other tissues provides a great advantage in tissue repair and regeneration studies. The fact that dental pulp tissue is a composite tissue consisting of fibroblast, endothelial cells, nerve cells, odontoblast, and osteoprogenitor and immune cells gives DP-MSCs a functional and unique feature (Ledesma-Martínez et al. 2016; Nuti et al. 2016). Ectodermal stem cells travel from the neural tube to the oral area during tooth formation, giving rise to OMT-MSCs. They eventually become mesenchymal cells at the end of the procedure (Aurrekoetxea et al. 2015). This distinguishes them from other neural crest cells in terms of biological features, making them ideal for therapeutic use.

In MSCs-based clinical therapies, strategies based on improving endogenous MSCs in the microenvironment have begun to emerge to ensure the engraftment and migration of transplanted cells (Grayson et al. 2015; Gao et al. 2017; Zhou et al. 2018). Since the huge efforts given to exogenous MSCs, it is critical to take use of endogenous MSCs, which dwell inside the tooth pulp and/or particular tissues and can self-renew and differentiate into different germ layers (Xia et al. 2018; Zhu et al. 2018). The number and activity of endogenous MSCs gradually decreases in post-natal development, resulting in a decrease in their natural repair abilities (Xia et al. 2018). Similarly, various chronic pathological conditions, i.e., diabetes mellitus, myocardial infarction, etc., and the process of physiological aging over time cause significant reduction in their functionality and reparability (Jiang et al. 2008; Haider 2018). Likewise, under inflammatory conditions such as pulpitis, periodontitis, osteoporosis, and implantitis, endogenous MSCs largely lose their differentiation ability and immunomodulatory properties (Xue et al. 2016; Di et al. 2018; Yang et al. 2017). Indeed, tumor necrosis factor-alpha (TNF-α) is a vital component of the inflammatory response, and it was reported that excessive levels and prolonged exposure to TNF-α are always associated with inflammatory diseases of the bone or tissue and lead to the death or impairment of endogenous MSCs' function.

Pharmacological approaches are also among the options used for the application of MSCs in bone tissue engineering and dentistry. The addition of pharmacological or biological agents to be incorporated into the structure of tissue scaffolding, membranes, composites, or filling materials for use in bone and dental tissue regeneration may be a good strategy for the recovery of endogenous MSCs' functionality, including survival, proliferation, differentiation potential, and paracrine

activity (Potu et al. 2009; Jeong et al. 2010, 2014; Li et al. 2010; Huang et al. 2012; Gu et al. 2015; Chen et al. 2016; Lee et al. 2016; Mendi et al. 2017a, b, 2019; Bourebaba et al. 2019). Considering these limitations of synthetic drugs, probiotics and/or their metabolites could be a new perspective in cellular therapies in regenerative medicine (Bourebaba et al. 2019; Cai et al. 2019).

The finding of oromaxillofacial tissue-derived mesenchymal stem cells, their biological roles, and comparisons with other tissue-derived MSCs, as well as probiotic bacteria and their relationships, are described in this review.

Identification, Isolation, and Biology of OMT-Derived Stem Cells

OMT cells were classified as epithelial and oral mucosal stem cells (EOM-SCs), cranial bone (CB) MSCs, mandibular bone marrow MSCs, and dental pulp MSCs (dental pulp MSCs, dental follicle (DF) MSCs, periodontal ligament (PDL) MSCs, and apical papilla (AP) MSCs) **(**Fig. 1**)** (Mendi et al. 2019).

Gronthos et al. (2000) were the first to report dental pulp stem cells. They used the same procedure that had previously been used to isolate and characterize iliac bone marrow MSCs (iBM-MSCs). They discovered a population of clonogenic cells within adult human tooth pulp. Cranial bone MSCs, epithelial and oral mucosal stem cells, and dental MSCs were all discovered using the same method **(**Figs. 1b and 2).

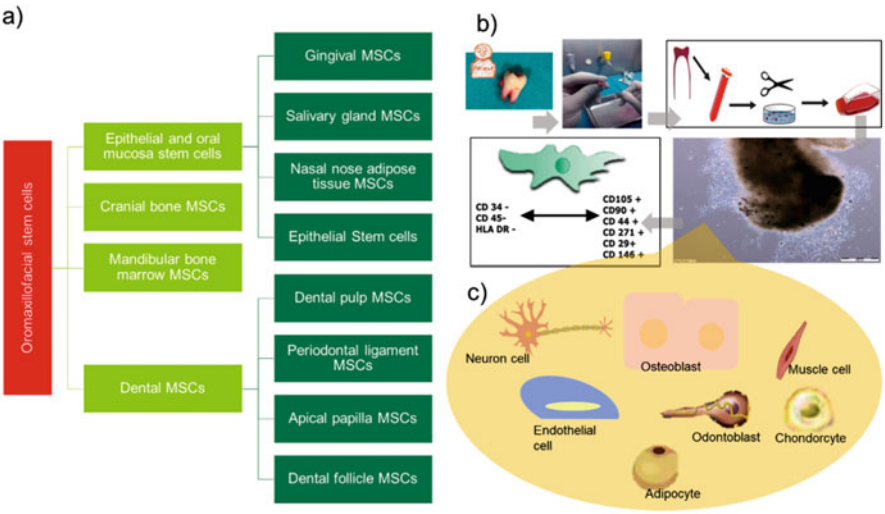

Fig. 1 **(a)** OMT mesenchymal stem cells classification, isolation, and identification. **(b)** Mandibular/maxillar bone marrow tissues or blood samples were removed and cultured using the explant culture method. Flow cytometry was used to sort the generated colonies. **(c)** The ability of OMT-derived MSCs to differentiate (Mendi et al. 2019)

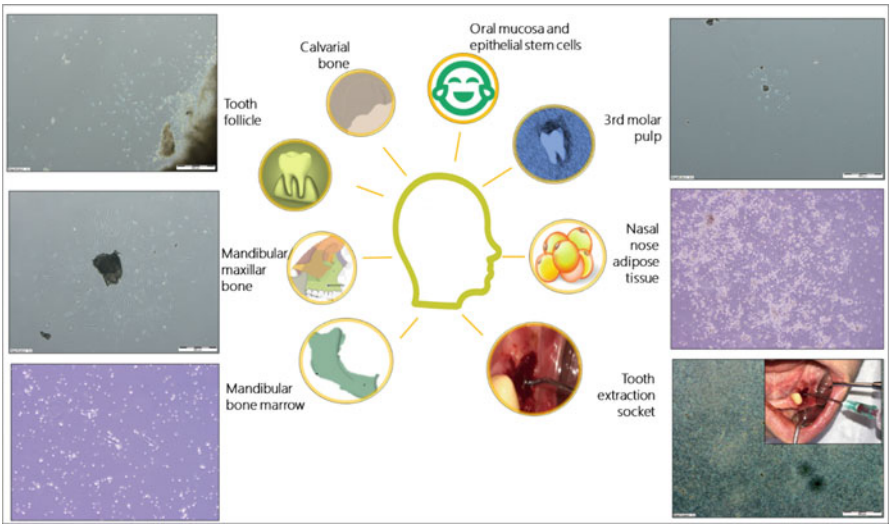

Fig. 2 Isolated OMT-MSCs. Our group had previously isolated MSCs from the third molar pulp, nasal adipose tissue, tooth extraction socket, mandibular bone marrow, cranial bone, and tooth follicle tissue (Mendi et al. 2019) (Olympos CKX 41, Japan)

Dental pulp is a composite tissue consisting of odontoblasts, interstitial fibroblasts, blood vessel networks, and nerve cells on the mineralized dentin surface (Sacchetti et al. 2007; Mendez-Ferrer et al. 2010; Bianco 2011). MSCs originating from DP bear similar surface markings as bone marrow MSCs accepted as reference. CD44, CD73, CD105, STRO-1, and CD146 are positive and also bear neural cell surface markers (Huang et al. 2009). On the other hand, there is a big gap to enrich pure MSCs (Keating 2012; Eleuterio et al. 2013; Liu et al. 2015; Ledesma-Martínez et al. 2016; Aghajani et al. 2016; Niehage et al. 2016; Werle et al. 2016).

OMT-MSCs have been reported to have functional and phenotypic differences from iBM-MSCs. Akintoye et al. (2006) reported that when comparing mandibular BM-MSCs isolated from the same individual and BM-MSCs of iliac origin, mandibular BM-MSCs showed higher proliferation rate and advanced osteogenic differentiation. In a study conducted by our group, it was observed that the adipogenic power of OMT-derived MSCs was lower than that of iliac-derived BM-MSCs (Mendi et al. 2017a, b).

It was also shown by our group that DP-MSCs have a shorter lag phase than iliac BM-MSCs and enter the logarithmic phase at higher cell counts (Mendi et al. 2017a, b). Our findings support the findings of Gronthos et al., who found that DP-MSCs, cranial bone MSCs, and epithelial oral mucosa MSCs colonize at a higher rate than iliac BM-MSCs (2000). The developmental condition of the various tissues can suggest this.

The mechanism in the interaction of OMT-MSCs with each other has not yet been elucidated. It was hypothesized that OMT-MSCs, like osteogenic tissues, have limited differentiation capacity, as they were originally thought to be specialized

tissues. It has been reported that OMT-MSCs have multidifferentiation power in tissue comparative studies published in recent years (Zhang et al. 2009; Wang et al. 2010; Moshaverinia et al. 2012, 2013, 2014).

The oral cavity consists of a flora containing more than 700 pathogenic and nonpathogenic microorganisms with different oxygen needs (Zhou et al. 2020). It's crucial to understand how bacteria in the flora affect MSCs in an inflammatory environment, as well as their effects on tissue repair. It is necessary to know the effects of existing bacteria on the stemness and potency of MSCs. It is known that there is a regression in the viability and differentiation of MSCs in the chronic inflammatory environment (Lee et al. 2009). The MSCs number is relatively sparse (Gao et al. 2018; Roddy et al. 2011). Such a microenvironment will, of course, influence MSCs clinical practice. Therefore, the interaction between OMT-MSCs and oral flora should be well-known.

In vitro and in vivo, MSCs have been shown to have anti-inflammatory and immunomodulatory properties. The effects of inflammation on OMT-MSC proliferation, migration, stemness, differentiation, and cytokine production have been studied in a variety of ways (according to Sensebé et al. 2010). Techniques to boost MSC proliferation, differentiation, and resistance to inflammation and oxidative stress are constantly being researched based on these beneficial features of MSCs. As a result, preconditioning strategies have attracted a lot of attention.

Preconditioning Strategies

MSCs originating from DP bear similar surface markings as bone marrow MSCs accepted as reference. CD44, CD73, CD105, STRO-1, and CD146 are positive and also bear neural cell surface markers. For example, many different osteo-inductive agents stimulate cell migration and proliferation and modulate immune response, including the stimulation of MSCs and/or osteo-progenitors in vitro and in vivo (Giannoudis et al. 2005; Govender et al. 2002). However, because they have a short half-life, bone tissue engineering requires either large concentrations or long-term administration (Itoh et al. 2001). It's also worth noting that their increased concentration could lead to enhanced osteoclastic activity and bone resorption (Kaneko et al. 2000). Therefore, it is important to develop alternative techniques and protocols to avoid these undesired effects.

In recent years, preconditioning strategies have come to the fore in order to increase the engraftment of MSCs in vivo or in clinical applications, to increase their survival in the inflammatory environment, and to differentiate them effectively (Haider and Ashraf 2012). Moreover, preconditioning approach also promotes their emigrational capacity, paracrine activity, and survival rate **(**Fig. 3**)** (Guo et al. 2020). Various stress conditions, including isolation from the issue source and in vitro expansion and harsh microenvironment (ischemia, hypoxia, and inflammation) post engraftment, cause extensive oxidative stress injury to the cells after engraftment at injured sites, which significantly reduces the survival of transplanted MSCs (Guo et al. 2020; Dissanayaka et al. 2020). Thus, different preconditioning strategies have

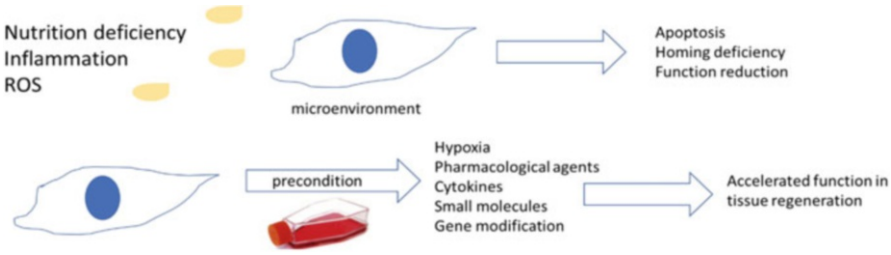

Fig. 3 MSCs face a harsh microenvironment that may induce their apoptosis or functional abrogation, such as poor differentiation potential, low adhesion, or attenuated proliferation

been developed to enhance MSCs functionality and tissue reparability post engraftment.

Among these, there are studies in vitro as well as in the experimental animal models to observe the effect of hypoxia in improving the migrational potential of MSCs. It has been observed that continuous or intermittent exposure to hypoxia or anoxia significantly promotes the differentiation and proliferation potential of MSCs. Similar effects have also been observed after preconditioning with different pharmacological agents, directing immunomodulatory properties by cytokine induction and regulation of differentiation and immune properties by gene modification (Jiang et al. 2006; Haider et al. 2008, 2009; Kim et al. 2009, 2012; Afzal et al. 2010; Lai et al. 2012).

Considering the direct and/or indirect relationship of OMT-derived MSCs with the oral flora, it should be considered that MSCs will also be affected by the effects of the bacterial flora on the immune response.

Among these bacteria, studies show that probiotics, which play a stabilizing role as a part of the intestinal flora, have similar effects on the oral flora and therefore are drawing significant attention by researchers.

Probiotics and their Health Benefits Associated with Gut Microbiota

Probiotics are one of the most remarkable functional foods to emerge in recent decades (Pray et al. 2013). In 2001, a joint committee of the United Nations Food and Agriculture Organization and the World Health Organization (FAO/WHO) defined probiotics as "live bacteria that bestow health advantages on the host when provided in suitable concentrations." The joint committee issued guidelines in 2002 to give a systematic strategy to evaluating probiotics, which included genetic and phenotypic strain identification, safety assessment, and functional analysis (FAO/WHO 2001, 2002).

Probiotics have been linked to a reduction in gastrointestinal pathogens, a reduction in cold and flu incidence, a reduction in colorectal cancer, improved lactose tolerance, and a significant reduction in the symptoms of inflammation-related

disorders (Salminen et al. 2005; Hörmannsperger et al. 2009; Leyer et al. 2009). It's worth noting that, despite a slew of health benefits linked to probiotics, the stated benefits can only be attributed to the strain in question, not the species as a whole or other probiotics. This is significant since some published reviews place a greater emphasis on the practical importance of individual probiotics to their higher taxonomic group than on strain specificity (Hill and Sanders 2013; Sanders et al. 2018).

Probiotic administration activates the immune system of the host, and as a result, some strains of probiotics can help to reduce inflammation in inflammation-related disorders including inflammatory bowel disease (IBD) (Kruis et al. 2004; Mogna et al. 2012). Probiotics can also help to regulate complex allergic inflammatory reactions brought on by allergy illnesses caused by a microbial imbalance (Isolauri et al. 2000; Sanders et al. 2013). A few well-designed trials have provided evidence for the usefulness of certain probiotic strains in treating individuals with atopic illnesses in the treatment of allergic disease. In a double-blind, randomized, placebo-controlled trial, *L. rhamnosus* GG was shown to prevent atopic eczema among children at high risk for atopic eczema. *L. rhamnosus* GG was administered prenatally for 2–4 weeks and postnatally for 6 months. The cumulative risk of eczema development was significantly reduced in patients as long as 7 years after the trial (Kalliomäki et al. 2001; Kalliomaki et al. 2007).

A 3-month trial involving 1,072 senior volunteers who drank probiotic dairy products containing *L. casei* DN-114 001 revealed that the *L. casei* strain is effective against respiratory infections (Boge et al. 2009). In comparison to the control group, the same strain also reduced common infectious illnesses, particularly upper respiratory tract infections (Guillemard et al. 2010).

Probiotics exert their health benefit with the multifactorial mechanism of action, including competing with microorganisms for nutrients and adhesion sites, producing antimicrobial peptides and organic acids, immunomodulation, and enhancing intestinal barrier (Shanahan 2010; Yan and Polk 2020). In a mouse model of acute colitis, *E. coli* Nissle 1917 reduced *Salmonella enterica* Typhimurium intestinal colonization by outcompeting the pathogen for iron, which is a limiting resource in this environment. They also discovered that *E. coli* Nissle mutants lacking in iron absorption can colonize the mouse gut but had no effect on *S. enterica* Typhimurium colonization (Deriu et al. 2013). By improving the gut barrier function, probiotics help to prevent pathogenesis. In IBD patients, the epithelial barrier has been disrupted. In mice with chemically induced colitis, *E. coli* Nissle 1917 inhibits leaky gut (Ukena et al. 2007). In a recent study, the function of the intestinal barrier was restored in an experimental mouse model of autoimmune encephalitis (Secher et al. 2017). The scientists discovered that oral therapy with *E. coli* Nissle 1917, rather than archetypal K12 *E. coli*, reduced the severity of experimental autoimmune encephalitis. The positive benefits were linked to a considerable decrease in pro-inflammatory cytokines along with a rise in the anti-inflammatory cytokine IL-10. Similarly, in mice with intestinal inflammation, VSL#3, a multiprobiotic formulation, enhances intestinal permeability (Corridoni et al. 2012).

Antimicrobial metabolites produced by probiotics can inhibit the growth of other bacteria (O'Shea et al. 2012) or encourage host cells to create antimicrobials (AMPs)

such as defensin, regIII, and lysozyme (Lebeer et al. 2010). AMPs are one of the most important components of the innate immune system, and they help shape the intestinal microbiota by activating the intestinal mucosal defense (Ostaff et al. 2013; Cunliffe 2003; Ganz 2003). Oral dosing of *L. gasseri* SBT2055, for example, stimulated IgA synthesis in the mouse small intestine (Sakai et al. 2014).

Immunomodulation is the most often hypothesized probiotic mode of action, which occurs when probiotic bacteria interact with mucosal immune cells or epithelial cells in the gut (Azad et al. 2018). Substantial evidence has accumulated to support the immunomodulatory effect of probiotic strains (Ishikawa et al. 2005; Ai et al. 2016; Garcia-Castillo et al. 2019). In a double-blind, placebo-controlled trial, lactobacilli strain was found to have an immunomodulatory impact, lowering the levels of IL-5, IL-6, IFN-g, and IgE in patients with seasonal allergic rhinitis and reducing the severity of allergic rhinitis symptoms (Ivory et al. 2008). In another study, daily lactobacilli strain ingestion for 3 weeks decreased natural killer cell activity in male smokers when compared to a placebo control group (Reale et al. 2012). In the gut of patients with ulcerative colitis, rectal administration of *L. casei* DG changes the mucosal microbiota and reduces the production of TLR-4 and inflammatory cytokines while increasing the expression of IL-10, an anti-inflammatory cytokine (D'Incà et al. 2011). Depending on the strain and the situation, probiotics may affect the innate and adaptive immune systems in both directions, acting as anti-inflammatory or pro-inflammatory agents (Rochat et al. 2007; Chiba et al. 2010; Shida et al. 2011). Because MSCs and probiotics have comparable functional characteristics, the investigation of MSCs-probiotic interaction is gaining traction in preconditioning studies.

Mesenchymal Stem Cells-Probiotic Interaction

The human microbiome offers potential treatment approaches including reversing or rebalancing the microbiome toward health, based on an increasing understanding of how the microbiome affects health and disease. There have been several research analyzing the variety of skin and gut microbial communities in health and sickness (Qiao et al. 2018) as well as the function of gut microbiota on intestinal stem cell activity for decades. However, nothing is known about the impact of microbiota on oral wound healing, alveolar bone regeneration, and OMT-derived MSC activity.

With the increase in resistance to antibiotics, the use of probiotics in oral health has come to the fore. In different studies involving dental caries, periodontal diseases, and halitosis, probiotic bacteria therapy was used, and positive results were obtained (Meurman 2005). Recently, studies have been focused on the effect of probiotic bacteria on oral wound healing and bone regeneration and mesenchymal stem cells (Han et al. 2020; Brandi et al. 2020).

Oral disorders are frequently caused by the invasion of pathogenic bacteria into the oral tissues, which disrupts the oral microbial flora balance and alters the oral mucosal immune response (Li et al. 2021). Probiotic bacteria, on the other hand, interact with epithelial cells (ECs), M cells, and dendritic cells in the intestinal milieu

through cell-cell contact or internalization of the bacteria or its components (cell wall, exopolysaccharides) (DCs). It increases IL-6 release from ECs and alters TNF-alpha and IFN-gamma secretion in MCs and DCs as a result of this interaction. At the same time, TGF-beta-induced T cell-independent transition from IgM to A on the surface of B cells is stimulated by IL-4 generated by mast cells (MACs) in combination with IL-6 released by ECs. IgA B cells' clonal growth is accelerated by IL-6, which also boosts the production of IgM, IgG, and IE antibodies. Th1 cells release pro-inflammatory IFN, which inactivates or kills viruses, tumor cells, and pathogenic germs. TNF and IL-2 are cytokines that promote phagocytosis and the formation of macrophages, NK cells, and cytotoxic T lymphocytes (Hoseinifar et al. 2019). It's possible that oral flora bacteria and gingival epithelial cells in the oral mucosa and OMT-derived MSCs in the connective tissue work in a similar way **(**Fig. 4**)** (Hoseinifar et al. 2019).

TLRs expressed by MSCs play an active role in directing the immune response by recognizing bacteria and in the migration, proliferation, and differentiation of MSCs. Long-term inflammation also negatively affects bone regeneration and wound healing through TLRs. In a study by Zhou et al. (2020) in which they compared TLR expression of G-MSCs, DP-MSCs, and BM-MSCs in inflamed and non-inflamed in vitro environments, DP-MSCs expressed TLRs 1–10 at low levels in the non-inflamed environment, while in the inflamed environment, observed increased expression of TLRs 2, 3, 4, 5, and 8. Similar results have been observed in other studies (Ciszek-Lenda et al. 2011; Xu et al. 2012; Tukenmez et al. 2019). Indeed our group demonstrated that *L. rhamnosus* ATCC9595 scaled down the expression of TLR4 and induced TLR2 expression when exposed to *P. gingivalis* stimulation on G-MSCs. ATCC 9595 induced CXCL5 in G-MSCs without

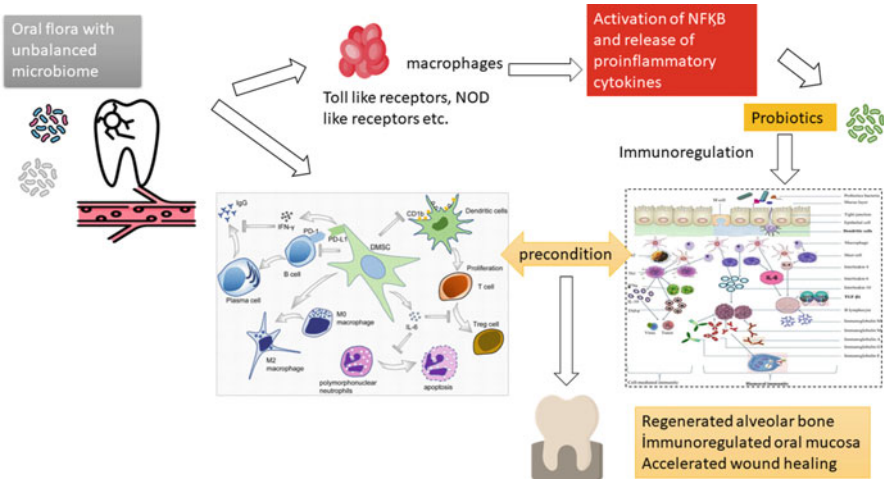

Fig. 4 Probiotic-OMT-MSCs interaction (the figure has been adopted from the Flaticon page) (Mendi et al. 2019)

triggering TLR-4 expression (Mendi et al. 2016). Although the bacteria in the oral flora and the immune signaling pathways they activate have an effect on the wound healing process, it is suggested that pathogenic bacteria delay wound healing (Jones et al. 2004). The common wound bacteria may hasten wound healing by increasing immune cell infiltration, granulation tissue creation, and collagen production, all of which are advantageous to the wound healing process (Su et al. 2018).

Studies reporting that the anti-inflammatory properties of probiotics have a positive effect on bone homeostasis are increasing. *L. rhamnosus* GG has been shown to inhibit bone loss by increasing the osteogenic differentiation of stem cells (Liu et al. 2020). From this point of view, the preconditioning of probiotic bacteria and MSCs for bone homeostasis is a new and up-to-date research topic.

Another study has revealed that LGG has a protective effect against radiation-induced intestinal epithelial damage via lipoteichoic acid. LGG lipoteichoic acid triggers adaptive immune response with macrophages and PGE2 from MSCs and supports epithelial stem cell niche to protect epithelial stem cells. Moreover, *L. rhamnosus* has been shown to improve human MSCs via its antioxidant effect, thus suggesting probiotics to be an alternative cell therapy agent (Ahadi et al. 2020).

By encouraging the migration of MSCs, LGG has been shown to protect intestinal epithelial cells against radiation damage. In an SD rat model, *Lactobacillus* also activates intestinal stem cells. Furthermore, there is emerging evidence that probiotics have a beneficial effect on bone homeostasis as a result of their anti-inflammatory properties. As a result, LGG may be able to prevent bone loss by increasing the osteogenic ability of stem cells (Liu et al. 2020).

Apart from these, there are also studies examining the behavior of mesenchymal stem cells by directing the host flora with probiotic microorganisms. Since, there is a balanced interaction between host immune system and gut microbiota, and this relationship is bidirectional which is necessary for optimal health and requires acknowledging that the disruption of this balance so-called dysbiosis can result in inflammatory and metabolic diseases such as inflammatory bowel disease (IBD), type 2 diabetes, obesity, arthritis, and asthma (Sommer and Bäckhed 2013; Ohno 2015; Hufnagl et al. 2020). Moreover, dysbiosis in oral microbiota leads to modulate epithelial markers such as β-catenin, E-cadherin and mesenchymal markers including fibronectin, and N-cadherin and impacts epithelial-mesenchymal transition (Chakraborti and Das 2020). In a study evaluating gut microbiota during mesenchymal stem cell-based therapy to improve acute liver injury in mice, they showed that change in the gut microbial population due to mesenchymal stem cell infusion to the liver could help liver repair by maintaining the gut mucosal homeostasis (Dong et al. 2019).

As a result, targeted changes in the gut microbiota could be used to prevent or treat diseases in humans. In Sprague-Dawley rats with collagen-induced arthritis, Li et al. studied the mechanism of human umbilical mesenchymal stem cells. They discovered that human umbilical mesenchymal stem cells, by modifying gut microbiota and immune response, had a therapeutic effect on rats with collagen-induced arthritis. Probiotics are one of the most prevalent ways for changing the gut's microbial composition.

In a study conducted by our group, it was observed that *L. rhamnosus*, one of the probiotic bacteria, preserved cell viability in gingival fibroblast culture in which an oxidative stress environment was created by using hydrogen peroxide, and it was suggested that it may have a protective effect against tissue damage. It is suggested that the examination of probiotic bacteria-connective tissue interactions in oral diseases will be an effective solution in periodontitis, mucosal wound healing, and immune oral diseases (Mendi and Aslim 2014). High EPS production of *B. breve* A28 strain was associated with antioxidant activity. In the results, it was observed that the oxidative stress effect of hydrogen peroxide application on gingival fibroblasts decreased by preventing iron chelation, DPPH radical scavenging, and plasma lipid peroxidation. Kim et al. (2020) demonstrated that *L. rhamnosus* promoted osteogenic and chondrogenic differentiation, reduced oxidative stress, and inhibited adipogenesis in MSCs (Kim et al. 2020).

Future Perspective and Conclusion

The oromaxillofacial tissue is a tissue rich in stem cells due to its composite structure, germ source, and neural crest origin cells. Mesenchymal stem cells abound in the oral and maxillofacial region. Primary teeth, third molars, and gingiva are easily accessible intraoral tissues, although they are frequently thrown as medical waste in clinics. Therefore, it is important for dentists to remember that tissues are a valuable source of stem cells during treatment in regenerative dentistry. Studies with probiotic bacteria in the dental clinic give varying results depending on individual oral flora and hygiene differences. Furthermore there is an apparent lack of sufficient in vitro models regarding the probiotic effect. On the other hand, it was highlighted that the activity of probiotic strains in vitro might not parallel similar behavior in vivo.

To further determine the role of probiotics preconditioned with MSCs in dentistry, large, well-designed, multicentered, controlled clinical trials and translational animal models are needed. The fact that not all *Lactobacillus* and *Bifidobacterium* species are equally beneficial should not be overlooked. Individual mechanism of each strain should be characterized, and the researchers should be aware of that the mechanism of action is dependent on the host characteristics, such as oral hygiene and nutrition habit.

Since different bacteria may have dominant effects in diverse genetic backgrounds and diseases involving their pathogenesis, researchers should prefer oral microbiome probiotics in clinical trials. To achieve more scientific evidence, further randomized controlled clinical trials with long follow-ups are necessitated. Determination of donor and patient histories, dietary patterns, and intestinal and oral flora compositions should be investigated in clinical applications combining cellular therapy and probiotic therapy. Probiotic microorganisms, on the other hand, don't just alter the microbiota. They can also influence MSC stemness by regulating immunological indices, epithelial permeability, and bacterial translocation, as well as supplying bioactive or regulatory metabolites. Thus, the interactions between

probiotic bacteria and MSCs could be explored by using comprehensive in vitro models that included mucosal stem cells.

As a result, the immunoregulatory effect of OMT-MSCs in co-culture with probiotic bacteria facilitates optimal tissue regeneration and can control tissue destruction. On the other hand, the functional properties and activities of probiotic strains vary between strains. It should be noted that not every probiotic strain has the same beneficial effect on health. Moreover, the probiotic strain isolated from the intestinal flora may have different effects in the oral flora due to the microhabitat difference. Probiotic strains in the oral microbiome need to be identified. In the selection and definition of oral probiotic strains, unlike intestinal probiotics, prevention of dental plaque formation, inhibition of periodontal pathogens, and regulation of cellular immune responses should be added. Furthermore, probiotic strain-oral mucosal cell interactions should also be investigated.

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Effects of 3D Cell Culture on the Cell Fate Decisions of Mesenchymal Stromal/Stem Cells

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Darius Widera

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Abstract

In vivo, all stem cells reside within distinct and defined microenvironments referred to as stem cell niches. Complex interactions between stem cells and their niche tightly regulate cell fate decisions. This affects live/death decisions, cellular senescence, and cell differentiation. Therefore, removing mesenchymal stromal/stem cells (MSCs) from their niche and cultivation within conventional flat, two-dimensional (2D) cell culture systems affects their cell fate and therefore also their regenerative potential. Nevertheless, routine MSC culture for research and therapy is still conducted in 2D. Three-dimensional (3D) cell culture is a state-of-the-art technology allowing expanding and differentiating MSCs under

D. Widera (✉)
Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, University of Reading, Reading, UK
e-mail: d.widera@reading.ac.uk

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more physiological conditions. This chapter will summarize current understanding of the impact of 3D cell culture on fundamental properties of MSCs including their proliferation, viability, cellular senescence, and differentiation. Furthermore, effects of 3D cell culture on the immunomodulatory and anti-inflammatory potential of MSCs will be evaluated.

Keywords

3D cell culture · Mesenchymal stem cells · Osteogenic differentiation · Adipogenic differentiation · Secretome

List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AD-MSCs	Adipose tissue-derived MSCs
ALP	Alkaline phosphatase
IL-10	Interleukin-10
IL-24	Interleukin-24
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LIF	Leukemia inhibitory factor
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MSCs	Mesenchymal stromal cells/mesenchymal stem cells
OCN	Osteocalcin
OPN	Osteopontin
PGE-2	Prostaglandin E2
TNF-α	Tumor necrosis factor-α

Introduction

MSCs are multipotent, fibroblast-like cells that can be readily obtained from various adult tissues, including the bone marrow, adipose tissue, and peripheral blood as well as from prenatal tissues such as amniotic fluid, umbilical cord, and placenta. Being multipotent adult stem cells originating from the embryonic mesoderm, their differentiation spectrum is limited to mesenchymal derivatives such as bone, fat, and cartilage cells (see Andrzejewska et al. (2019) for review). However, after their initial discovery by Alexander Friedenstein (Friedenstein et al. 1968, 1970, 1974), several reports suggested that MSCs might have an even higher differentiation potential and an intrinsic ability to cross the germ layer boundaries. In this context, differentiation of MSCs into neural and neuronal derivatives has been claimed in multiple independent studies (Jiang et al. 2002; D'Ippolito et al. 2004; Takeda and Xu 2015; Tropel et al. 2006; Scuteri et al. 2011). Notably, a vast majority of these reports relied heavily on nonfunctional data, including morphology and marker

expression without providing a direct side-by-side comparison to neural and neuronal cells. Nevertheless, it is noteworthy that MSCs can display neuron-like morphology in response to cellular stress. Moreover, they have been shown to express neuronal markers even after differentiation into osteogenic and adipogenic fate (Foudah et al. 2013) in vitro suggesting that this might be an in vitro *cell* culture artifact and not a result of functional differentiation across a germ layer boundary.

This view is also supported by several studies showing that MSCs do not differentiate and integrate if transplanted into brain tissue in vivo. In addition to reports suggesting that MSCs can undergo an ectodermal fate, it has been postulated that MSCs could differentiate into endodermal pancreatic islet-like cells (D'Ippolito et al. 2004; Zanini et al. 2011). Notably, also these reports provided no functional data supporting this claim. Nevertheless, over 1250 clinical trials involving MSCs have been registered on the ClinicalTrials.gov database with a wide indication profile including diabetes, cardiovascular disorders, in addition to musculoskeletal symptoms. It is noteworthy that MSCs are believed to be immunoprivileged making them ideal candidates for allogenic transplantation in many acute conditions such as heart infarct, where isolation and expansion of autologous cells is too time-consuming and cumbersome (Atoui et al. 2008a). Indeed, transplantation of allogenic and autologous MSCs has been shown to alleviate symptoms of a broad spectrum of diseases and symptoms including liver cirrhosis (affected germ layer: endoderm) (Kharaziha et al. 2009), severe ischemic heart failure (affected germ layer: mesoderm) (Mathiasen et al. 2015; Kalou et al. 2021), and progressive multiple sclerosis (affected germ layer: ectoderm) (Connick et al. 2011). This discrepancy between the promising preclinical and clinical in vivo data and the lack of cross germ layer differentiation in vitro can be explained by the nowadays widely accepted mode of action of MSCs, which is mediating paracrine bystander effects rather than a consequence of engraftment and differentiation (Sharma et al. 2014; Lee et al. 2009; Zanotti et al. 2013; Timmers et al. 2007; Gnecchi et al. 2005, 2006). The paracrine activity of the transplanted cells also promotes mobilization of the intrinsic stem cells from bone marrow for their participation in the repair process (Haider et al. 2008; Haider and Aziz 2017). Given their paracrine activity containing both soluble and insoluble constituents, cell-free therapy using MSCs-derived paracrine factors is fast emerging as an alternative to cell-based therapy (Haider and Aslam 2018; Haider and Aramini 2020).

Thus, this chapter will focus mainly on the influence of 3D cell culture on osteogenic, adipogenic, and chondrogenic differentiation of MSCs, in addition to an assessment of the effects of cultivation in 3D on viability, proliferation, cellular senescence, and anti-inflammatory and immunomodulatory potential of MSCs' secretome.

3D Cell Culture of MSCs

In the developing embryo and in the adulthood, stem cells reside in defined distinct microenvironments referred to as endogenous niches (Brook and Gardner 1997; Frenette et al. 1998; Johansson et al. 1999). The current understanding of these

stem cell niches is that it not only includes stem cells but also involves cell-cell contacts, the extracellular matrix (ECM), as well as diverse biochemical and biophysical signals. These factors dictate the fate of the stem cells within the niche (Schofield 1978; Collins et al. 2005; Colmone et al. 2008; Conboy et al. 2005). Thus, proliferation, viability, differentiation, and the paracrine activity of stem cells highly depend on extrinsic factors within the microenvironment. As a result, the regenerative potential of stem cells is tightly linked to the homeostasis of its given niche. In vivo, MSCs reside in their respective niche (e.g., the perivascular niche for MSCs within the bone marrow). Their fate is regulated by integrating direct, paracrine, and endocrine signals received from various other cell types, other MSCs, extracellular matrix, and extrinsic and autocrine signaling molecules. In light of the complexity of these signaling networks, it is not surprising that removing stem cells from their niche leads to a profound disturbance of the signaling cascades that regulate cell fate.

Nevertheless, most basic and translational stem research is still conducted as conventional two-dimensional (2D) cell culture. Notably, it has been clearly shown that 2D cell culture results in an unnatural and forced apical cell polarity and changes their proliferation, migration, and differentiation capability. To address this, different strategies have been applied to recreate a more physiological microenvironment in vitro.

One of the most prominent and oldest techniques to cultivate stem cells three-dimensionally has been firstly established in 1992 (Reynolds and Weiss 1992). In this pioneering study, Reynolds and Weiss used a serum-free cultivation medium to enrich neural stem cells from enzymatically and mechanically digested adult mouse brain tissue. This resulted in self-aggregation of neural stem cells into spheroids and proliferation as self-adherent clusters referred to as neurospheres. In the following, similar protocols have been applied to other cell types, including various cancer cells (tumorispheres), embryonic stem cells (embryoid bodies), and MSCs. While being cost-effective and easy, this method is limited by the unequal distribution of nutrients and growth factors within the spheres and a lack of physiological tissue geometry.

An alternative to spheroid cultures is the use of scaffolds that can be solid or provided as hydrogels (Fig. 1). This scenario is the most common strategy to cultivate mammalian stem cells within a 3D environment. Here, the endogenous stem cell niche is mimicked even more closely by matrices or matrix-like structures (Lee et al. 2008).

To overcome the limitations of the conventional 2D cell culture, different 3D cultivation methods have been applied to MSCs (reviewed in Bicer et al. (2021)). Among others, MSCs have been successfully expanded as spheroids (Fig. 2a), and cultivated in 3D within various 3D hydrogels, including alginate hydrogels (Ho et al. 2016), collagen-based matrices (Lund et al. 2009), fibrin-poly(ester-urethane) scaffolds (Gardner et al. 2017), bacteria-derived cellulose (Favi et al. 2013), methylcellulose (Yamaguchi et al. 2014), nano-fibrillar cellulose (Azoidis et al. 2017; Sheard et al. 2019), and the chondrosarcoma (Fig. 2b, c) derived Matrigel™ (Yu et al. 2018).

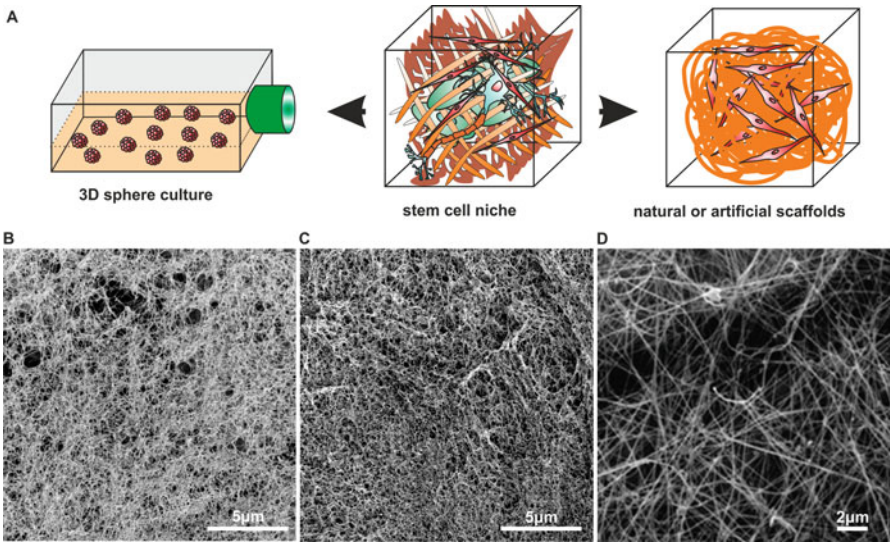

Fig. 1 (**a**) **Schematic view of common 3D methods for stem cell cultivation.** In their niche, stem cells reside in a distinct microenvironment which includes biochemical signals and complex 3D architecture providing physicomechanical cues. Removal from the niche, stem cells can be cultivated as conventional 2D monolayers or be propagated as self-adherent 3D spheres (left). Alternatively, cells can be cultivated on the surface or embedded within 3D scaffolds (right). (**b**–**d**) Microscopic appearance of nanofibrillar cellulose (**b**, unpublished), anionic nanofibrillar cellulose (**c**, (Sheard et al. 2019)), and blood plasma-derived fibrin hydrogels (**d**, (Greiner et al. 2011))

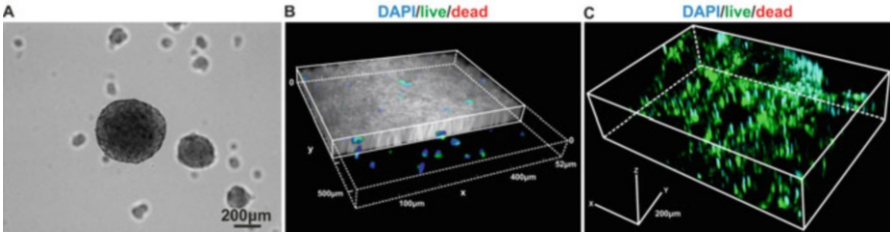

Fig. 2 **Different approaches to 3D cultivation of MSCs.** (**a**) MSCs derived from human dental pulp were cultivated in serum-free medium supplemented with FGF-2 resulting in formation of self-adherent spheroids (unpublished). (**b**) Human AD-MSCs were embedded within a 0.2% nanofibrillar cellulose hydrogel and stained with calcein (living cells), ethidium homodimer-1 (dead cells), and DAPI (nuclei). (Data from Azoidis et al. (2017)). (**c**) AD-MSCs were cultivated in 0.2% anionic nanofibrillar cellular and stained with calcein, ethidium homodimer-1, and DAPI

In addition, MSCs have been cultivated on a variety of stiff scaffolds which are beyond the scope of this chapter. In the following, we will focus on the most common approaches to 3D cultivation of MSCs – the spheroid culture and 3D cell culture within scaffolds.

Impact of 3D Cell Culture on Cell Fate Decisions of MSCs

Two-dimensional (2D) cell culture is still widely used to expand stem cells and to produce other cell-based therapeutics. This usually involves plating cells on flat plastic or glass surfaces. However, it is worth noting that the endogenous stem cell niche is not flat and two-dimensional. Therefore, removing stem cells from their niche not only changes the biochemical signals the cell receives but also mechanophysical aspects of the cell surroundings. This results in unnatural cell polarity and affects the proliferation kinetics, differentiation potential, and the profile of paracrine signals the cell is secreting.

It has been demonstrated that the cultivation of MSCs in traditional 2D cell culture is associated with a loss of multipotency and induction of premature cellular senescence (Turinetto et al. 2016). In addition, conventional cell culture has been suggested to induce chromosomal aberrations within the MSCs' genome (Ben-David et al. 2011; Bara et al. 2014). These artifacts can be avoided by the cultivation of MSCs as modern 3D cell cultures. Indeed, MSCs have been cultivated in a wide range of 3D cell culture systems including spheroids (Redondo-Castro et al. 2018; Bartosh et al. 2010) (reviewed in Cesarz and Tamama (2016)), stiff substrates, including PEDOT: PSS (Shahini et al. 2014), as well as different hydrogels including alginates (Ho et al. 2016), collagen (Lund et al. 2009), Matrigel (Yamaguchi et al. 2014), and different forms and preparations of cellulose (Favi et al. 2013; Cochis et al. 2017; Azoidis et al. 2017; Sheard et al. 2019).

However, the effects of 3D cell culture on the fundamental properties of MSCs seem to be at least partly dependent on the nature of the culture system. In the following, this chapter will discuss how different 3D cell culture systems affect MSC viability, proliferation, differentiation, and paracrine activity in comparison to respective 2D cell culture controls.

Viability and Proliferation

The impact of 3D cell culture on cell viability and proliferation has been extensively studied in cancer cells. Interestingly, the effects of 3D cell culture seem to be cell-type specific and technique-dependent, with some reports suggesting positive effects while others reported negative effects reviewed by Edmondson et al. (2014). A possible explanation is that the applied 3D culture methods varied largely with some reports utilizing scaffold-free tumorisphere cultures and other applying more advanced solid scaffolds or hydrogels. Overall, the cultivation of cancer cells as tumorispheres has frequently been associated with decreased proliferation and viability while 3D hydrogels have been shown to perform equally or be even superior compared to 2D cell culture (reviewed in Mirbagheri et al. (2019)). As proliferation and viability are critical for the expansion of MSCs in a clinical scenario, evaluating the impact of 3D cell culture technology on these parameters is paramount for the successful translation of basic research into the clinics.

While there is some evidence that spheroid culture of other cell types, including cancer cells and neural crest-derived stem cells reduces proliferation and viability (Hauser et al. 2012), the picture is more complex in case of MSCs. Since there is evidence for positive (Lee et al. 2016; Zhao et al. 2015), neutral (Domnina et al. 2018), or even adverse effects on proliferation (Sun et al. 2018; Baraniak and McDevitt 2012; Tsai et al. 2015), the impact of 3D spheroid culture in MSCs is still controversial. Overall, the adverse effects could be results of non-homogeneous nutrient and oxygen supply and impaired waste product diffusion in the core of the spheres (reviewed in Cesarz and Tamama (2016)). In contrast, the positive effects could be explained by more physiological cultivation conditions at the margin of the spheres compared to flat 2D cell culture. The picture gets even more complex when assessing the effects of cultivating MSCs within hydrogels. For several 3D scaffolds, including gelatine, poly lactic-co-glycolic acid, and chitosan, no evident effects on viability and proliferation have been reported (Lo et al. 2016). However, especially collagen hydrogels appear to exert different effects depending on the preparation of the hydrogel and the origin of the cells. While some studies suggest that collagen does not impact proliferation and viability of human MSCs (Lo et al. 2016), other reports indicate that the same scaffold increases proliferation of rat bone marrow MSCs (BM-MSCs) (Han et al. 2012). Similarly, there are contradicting data on the effects of polycaprolactone.

Increased viability and proliferation of minipig MSCs has been described in 2013 (Rampichova et al. 2013), while a recent study did not reveal any changes of proliferation and viability of human adipose tissue-derived MSCs (AD-MSCs) in 3D printed polycaprolactone/tricalcium phosphate compared to 2D controls (Park et al. 2018). Conversely, sprayed micro-fiber polycaprolactone has been suggested to increase the proliferation of human BM-MSCs, while electrospun variants of the same scaffold decreased their proliferation rate (Brennan et al. 2015). Similarly, poly (l-lactic acid) has been suggested to reduce the viability of BM-MSC, while the same scaffold substituted with hydroxyapatite increased it (Persson et al. 2018). In contrast, data from an independent lab indicated that a blend of poly (l-lactic acid) and type I collagen reduces the proliferation of human MSCs (Nguyen et al. 2012).

Similarly, the effects of the cultivation of MSCs in 3D nanofibrillar cellulose seem to differ depending on the concentration of the gel and its charge. While low concentrations (0.2%) did not change the proliferation rate of BM-MSCs, AD-MSCs, and palatal MSCs, higher concentrations (0.5%) resulted in a decrease in viability and proliferation (Azoidis et al. 2017).

However, anionic nanofibrillar cellulose has been shown to increase the viability of human AD-MSCs even at higher concentrations (Sheard et al. 2019) (Fig. 3).

Overall, there is no clear trend regarding the effects of 3D cultivation on the viability and proliferation of MSCs. The outcome is evidently dependent on many parameters, including the method of preparation of the scaffold, its concentration, as well as the species and tissue of origin of the cells used in the 3D cell culture system. Thus, adapting existing cultivation protocols to 3D cell culture requires careful monitoring of both parameters to establish optimized cultivation parameters for each scaffold and each cell type.

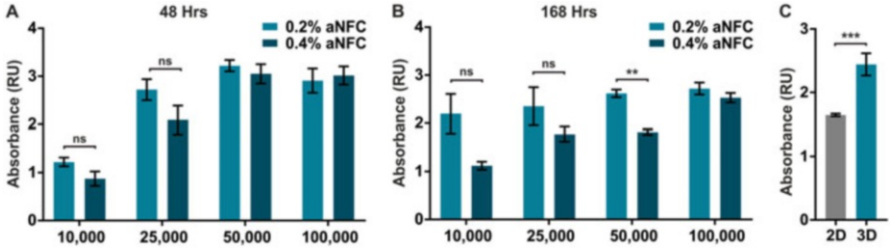

Fig. 3 Viability of MSCs in 3D anionic fibrillar cellulose varies depending on the seeding density and is superior compared to 2D controls. XTT viability analyses of different concentrations of AD-MSCs seeded within different densities of aNFC hydrogels show high viability after 48 h and 1 week (Sheard et al. 2019)

Cellular Senescence

Traditional 2D cell culture of flat, stiff substrates, such as plastic and glass, has been shown to increase cellular senescence of multiple cell types, including but not limited to MSCs. There is growing evidence in the literature suggesting that 3D cell culture might reduce senescence. This could be explained by the fact that the 3D environment closely mimics the endogenous niche of the MSCs while 2D enforces an unnatural apical polarity.

The impact of the most basic 3D cell culture system, the spheroid culture, on MSC senescence is ambiguous. It has been reported that spheroid cultures of AD-MSCs increase their replicative potential whilst reducing their senescence (Cheng et al. 2013; Younis et al. 2018). Similarly, human endometrial MSCs cultivated as 3D spheroids showed reduced levels of cellular senescence in response to heat shock and H_2O_2 compared to 2D controls (Domnina et al. 2020). Interestingly, although the spheroid MSCs did not show signs of senescence, the authors observed a decreased viability compared to 2D cell culture. Conversely, another study indicated that cellular senescence increases in MSC-spheroids, especially at late time points (Whitehead et al. 2020). These contrasting results could be explained by the nature of the spheroid culture system. At early stages, all cells within the clusters have sufficient access to nutrients from the environment and disposal of waste products of metabolism if not yet hampered by surrounding cells. However, once a critical size of the spheres is reached, lack of nutrients and accumulation of toxic waste initiate focal necrosis in the core of the clusters and cellular senescence in adjacent cells.

In contrast, this phenomenon is mainly avoided if 3D cell culture is conducted using hydrogels as scaffolds. A study by Younis and colleagues revealed that TNF-α-induced cellular senescence was largely reduced in MSC-like human periodontal fibroblasts cultivated in 3D methylcellulose hydrogels (Younis et al. 2018) when compared to 2D cultivated control cells. In accordance with these finding, a more recent report has provided clear evidence for a significant reduction in senescence-associated β-galactosidase activity in human adipose-derived MSCs cultivated in commercial polysaccharide hydrogels. Interestingly, the authors also

reported an overall increased telomerase activity when MSCs were subjected to 3D cell culture (Yin et al. 2020).

Overall, both spheroid and scaffold-based 3D cell cultures appears to reduce the cellular senescence of MSCs from different origins. However, sphere-based 3D cell culture seems more suitable for short-term expansion of MSCs since the positive effects on cellular senescence are less obvious at the late stages of the culture.

Osteogenic Differentiation

Osteogenic differentiation is the most widely studied cell fate of MSCs (Shima et al. 2015; Matta et al. 2019; Zhang et al. 2021). This is highlighted by the vast amount of research studies focused on MSC-based bone regeneration and numerous clinical trials exploiting the potential of MSCs to undergo this differentiation path. Interestingly, the data regarding the effects of 3D cell culture on osteogenic differentiation of MSCs are much more coherent than data sets focused on its influence on proliferation, viability, and senescence (Fig. 4). Overall, both scaffold-free and scaffold-based 3D cell culture systems appear to increase the osteogenic potential of MSCs. In this context, rat BM-MSCs expanded as spheroids showed a higher Osterix expression, increased ALP activity, and higher mineralization compared to 2D controls (Yamaguchi et al. 2014). In accordance with this report, the cultivation of

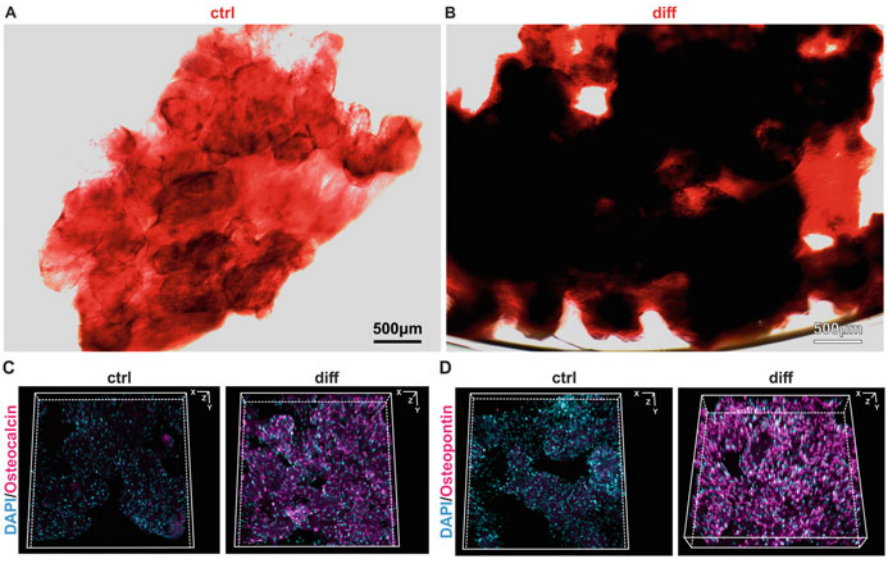

Fig. 4 Osteogenic differentiation can be conducted in 3D. (**a**, **b**) Alizarin Red S staining of human AD-MSCs subjected to osteogenic differentiation in anionic nanofibrillar cellulose hydrogels. Please note a more intense staining in cells cultivated in osteogenic differentiation medium. (**c**, **d**) 3D osteogenic differentiation of AD-MSCs in anionic nanofibrillar cellulose increase expression of osteocalcin and osteopontin. (Data from Sheard et al. (2019))

mouse BM-MSCs as scaffold-free spheroids resulted in fivefold higher levels of calcium deposition compared to 2D cultures exposed to the same osteogenic differentiation medium (Baraniak and McDevitt 2012).

Similarly, scaffold-based 3D cell culture systems have been consistently shown to increase the osteogenic potential of MSCs from different species and tissues. Human AD-MSCs cultivated in 3D rat tail collagen hydrogels revealed increased the expression of osteogenic markers, including type I collagen, osteopontin (OPN), and osteonectin (OCN) (Sefcik et al. 2008). Consistently, rat BM-MSCs expanded in 3D collagen scaffolds expressed higher levels of OCN and OPN as well higher levels of calcification compared to osteogenically differentiated cells in 2D (Han et al. 2012). In accordance with these reports, human MSCs of undefined origin showed higher levels of OCN and OPN and increased mineralization when subjected to osteogenic differentiation in blended scaffolds composed of collagen and electrospun poly(l-lactic acid) (Nguyen et al. 2012). Similarly, a significant increase in calcium deposition was observed in human BM-MSCs cultivated in 3D scaffolds composed of pure poly (l-lactic acid) (Persson et al. 2018). Similarly, differentiation of human BM-MSCs in Engelbreth-Holm-Swarm mouse sarcoma-derived Matrigel increased the ALP activity (a marker of osteogenic differentiation) and led to higher mineralization levels compared to cells differentiated in 2D (Yu et al. 2018).

Consistent with the results discussed above, scaffold-based 3D cell culture in 3D poly (ε-caprolactone) has been also shown to increase osteogenesis of minipig BM-MSCs (Rampichova et al. 2013), and human fetal and adult BM-MSCs (Shekaran et al. 2015; Brennan et al. 2015). Finally, alginate and gelatine blends increased the levels of osteogenic differentiation of AD-MSCs and BM-MSCs (Lo et al. 2016; Wang et al. 2016).

Adipogenic Differentiation

In 2019, Zohora and colleagues analyzed gene expression patterns of human AD-MSCs differentiating toward the adipogenic fate in 2D and embedded within hydrogels composed of self-assembling peptides. Although no comparison of the levels of lipid accumulation between 2D and 3D has been performed, the authors noted that the expression of genes regulating adipocyte-like cell properties was more consistent in 3D-cultivated cells (Zohora et al. 2019). Earlier reports also suggested that mouse BM-MSCs cultivated as 3D spheroids accumulate higher levels of lipids than 2D controls when subjected to adipogenic differentiation (Baraniak and McDevitt 2012; Bae et al. 2017). Interestingly, a recent study by Niibe and colleagues showed that the adipogenic potential of mouse and human MSCs pre-cultivated as adherent 2D monolayers lost as a result of long-term culture can be restored by transferring the cells into shaken 3D sphere culture systems (Niibe et al. 2020). Moreover, it has been shown that human AD-MSCs cultivated within polycaprolactone hydrogels accumulate higher levels of lipids and have a more mature adipocyte phenotype compared to their 2D cultivated counterparts. In contrast, Zou et al. reported in 2017 that the cultivation of human AD-MSCs in gelatine-based hydrogels does not significantly change lipid accumulation and the expression

of the adipogenic marker FABP-4 (Zou et al. 2017). Nevertheless, a vast majority of studies indicate that similar to the osteogenic potential, the adipogenic differentiation potential is increased in 3D as well (Miyamoto et al. 2016).

Chondrogenic Differentiation

As the chondrogenic differentiation of MSCs is mostly conducted as highly compact 3D pellets, a side-by-side 2D vs. 3D comparison of chondrogenic differentiation potential is challenging. Nevertheless, Merceron et al. compared chondrogenic differentiation of human AD-MSCs in 3D hydroxypropylmethyl cellulose hydrogels to differentiation in 2D. The authors provided clear evidence that the levels of chondrogenic differentiation are increased if chondrogenesis is conducted in 3D. Interestingly, thermo-reversible 3D methylcellulose-based hydrogels have also been shown to allow upscaling compatible chondrogenic differentiation of human BM-MSCs in bioreactors (Cochis et al. 2017). A direct comparison of chondrogenic differentiation of AD-MSCs as 3D pellets and within collagen I scaffolds indicated that scaffold-based culture might indeed result in a higher chondrogenic potential as evidenced by higher levels of type II Collagen, Aggrecan, and Sox9 as well as by a more intense Alcian blue staining. However, as mentioned above, the nature of the standard differentiation protocols makes assessing the impact of 3D cell culture on chondrogenic differentiation challenging.

MSC-Secretome, Immunomodulation, and Anti-Inflammatory Potential

Paracrine "bystander" effects are nowadays believed to be the primary regenerative mode of action for MSCs. This is especially important because MSCs exert beneficial effects on the regeneration of non-mesodermal tissues despite their lack of differentiation capacity into these cell types. Paracrine factors within the MSCs secretome mediate their beneficial effects mainly by reducing the levels of inflammation, modulating immune cells, increasing the levels of local angiogenesis, and inducing proliferation of endogenous tissue-resident progenitor and stem cells.

Consequently, evaluating the effects of 3D cell culture on the anti-inflammatory and immunomodulatory potential of MSCs is essential for a holistic analysis of their regenerative potential.

Overall, although very few reports suggest a reduction of the anti-inflammatory and immunomodulatory potential of MSCs as a result of 3D culture as spheroids (Burand et al. 2020), most studies provide strong evidence that secretomes produced by MSCs in 3D have an anti-inflammatory and immunomodulatory potential that is superior to their 2D cultivated counterparts. In this context, it has been demonstrated that AD-MSCs spheroids increase the expression and secretion of the pro-angiogenic vascular endothelial growth factor (VEGF) compared to 2D (Cheng et al. 2013). In addition, Bartosh et al. showed that spheroid cultures of BM-MSCs secrete higher levels of IL-24, which is a widely known anti-inflammatory

cytokine (Bartosh et al. 2010). The authors also revealed that spheroid BM-MSCs could more efficiently suppress LPS-induced inflammation compared to 2D cultivated cells.

Similarly, human MSC spheroids showed increased levels of IL-24 secretion (Frith et al. 2010). In a more recent study, Follin et al. demonstrated that MSC cultivated as spheroids secrete higher levels of VEGF than control 2D cultures (Follin et al. 2016). A similar increase of VEGF secretion has been reported in 3D spheroid cultures of human BM-MSCs (Redondo-Castro et al. 2018). In addition to the secretion of pro-angiogenic factors and reduction of inflammation, a pronounced increase of immunomodulatory activity has been observed in 3D cultivated MSCs. Briefly, MSCs cultivated as spheroids had higher potential to polarize macrophages toward the anti-inflammatory M2 phenotype. This has been attributed to increased secretion of prostaglandin E2 that mediated the macrophage polarization (Bartosh et al. 2013; Ylostalo et al. 2012). In addition to spheroid cultures, MSCs cultivated in hydrogels appear to produce secretomes with a higher regenerative potential than MSCs cultivated in 2D.

It has been reported that AD-MSCs cultivated in 3D bovine collagen/polyethylene glycol and collagen/low-molecular-weight hyaluronic acid hydrogels mediated higher levels of secretome-mediated neuroprotection of SH-SY5Y neuroblastoma cells following 6-hydroxydopamine insult compared to 2D cultivated controls (Chierchia et al. 2017). Similar to the changes in secretome profiles of spheroid cultured MSCs, scaffold-based 3D culture increased the secretion levels of HGF, VEGF, stromal cell-derived factor (SDF), and FGF-2 in AD-MSCs (Lee et al. 2016; Kim et al. 2013; Young et al. 2018). A recent study revealed that 3D cultivation of human BM-MSCs in electrospun gelatine/polycaprolactone scaffolds results in increased secretion of FGF-2, IL-6, VEGF, and HGF. Importantly, the authors also demonstrated that the secretomes from 3D cultivated cells were more efficient in corneal wound healing (Carter et al. 2019). Similarly, 3D cultivated umbilical cord MSCs secreted higher levels of IL-10, LIF, FGF-2, I-309, SCF, and GM-CSF than cells cultivated in 2D. Moreover, the same study showed a superior regenerative and anti-inflammatory potential in a rat in vivo arthritis model (Miranda et al. 2019).

Overall, 3D cell culture has been demonstrated to increase the regenerative potential of MSC secretomes independent of the nature of the cell culture system, suggesting that adapting 3D cell culture for cell expansion might be a promising step in improving the efficacy of MSC-based cell-free therapeutics (Kusuma et al. 2020; Bou-Ghannam et al. 2021).

Conclusions

Within their endogenous niche, the fate of MSCs is dictated by a complex network of signals, including cell-cell communication, geography of the niche, and interactions with the extracellular matrix. 3D cell culture mimics the niche more closely than flat, 2D cell culture. This chapter evaluated how 3D cell culture affects cell viability, senescence, differentiation, and paracrine potential of MSCs. As summarized above, the effects of 3D cell culture on cell viability and proliferation depend on the nature of

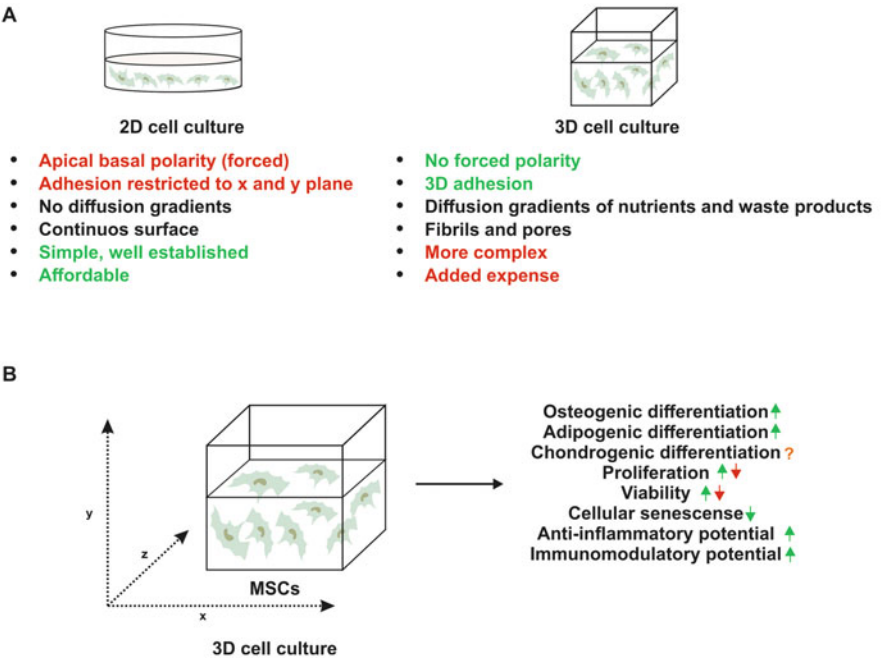

Fig. 5 **Effects of 3D cell culture on the cell fate of MSCs** (**a**). Advantages and drawbacks of 3D cell culture compared to traditional 2D cell cultivation. Advantages are highlighted in green, drawbacks in red, inconclusive aspects in yellow (**b**). 3D cell culture increases osteogenic and adipogenic differentiation potential of MSCs while there is not enough evidence to address the question how it affects chondrogenic differentiation. While there are conflicting reports on the impact of 3D cell culture on proliferation and viability, it decreases cellular senescence of MSCs and increases their anti-inflammatory and immunomodulatory potential

the applied cell culture technique and the origin of the MSCs. In contrast, the effects of 3D cultivation of cellular senescence, differentiation capability, and immunomodulatory and anti-inflammatory capacity are positive independent of the applied technique or scaffold (Fig. 5). Thus, going 3D might ultimately pave the way for better stem cell therapies and cell-free, MSCs-based therapeutics. Additionally, strategies based on physical, chemical (i.e., growth factor treatment), pharmacological, or genetic engineering as well as subcellular preconditioning could further augment the effects of 3D culture on MSCs in terms of survival, proliferation, differentiation, and paracrine potential (Lu et al. 2009, 2010; Kim et al. 2009; Afzal et al. 2010).

Cross-References

► Extracellular Vesicles Derived from Mesenchymal Stem Cells
► Human Mesenchymal Stem Cells: The Art to Use Them in the Treatment of Previously Untreatable

- ▶ Mesenchymal Stem Cells
- ▶ Mesenchymal Stem Cell Secretome: A Potential Biopharmaceutical Component to Regenerative Medicine
- ▶ Regenerative Medicine Applied to the Treatment of Musculoskeletal Pathologies
- ▶ Response of the Bone Marrow Stem Cells and the Microenvironment to Stress

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Part II

Cell-Based Therapy Approach: Non-Mesenchymal Stem Cell-Based Therapy

The Function of Stem Cells in Ocular Homeostasis

20

S. Amer Riazuddin, Shahid Y. Khan, and Muhammad Ali

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Abstract

Stem cells are defined as precursor cells with capabilities of self-renewal and the potential to differentiate into any type of specialized cells in the human body. Stem cells are categorized into two main types: pluripotent stem cells and adult stem cells. The former are derived from an embryo or can be generated by reprogramming and the latter from somatic tissues. The pluripotent stem cells can differentiate into three germ layers and tissue-specific stem cells, whereas the adult stem cells residing in somatic tissues can differentiate into specific cell lineage depending on the tissue environment. Multiple studies have characterized

S. A. Riazuddin (✉) · S. Y. Khan · M. Ali
The Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA
e-mail: sriazud1@jhmi.edu; skhan59@jhmi.edu; mriaz1@jhmi.edu

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adult stem cells in many human tissues, including the eye, and in recent years, there have been many reports highlighting the critical role of adult stem cells in maintaining the natural homeostasis of the eye. This chapter reviews the various populations of adult stem cells that exist in multiple compartments of the eye and their critical role in regeneration and repairability during ocular damage and/or disease.

Keywords

Cornea · Embryonic stem cells · Eye · Homeostasis · Induced pluripotent stem cells · Ocular · Pluripotent · Reprogramming · Retina · Stem cells

Abbreviations

AMD	Age-related macular degeneration
ESCs	Embryonic stem cells
iPSCs	Induced pluripotent stem cells
RP	Retinitis pigmentosa

Introduction

Pluripotency, the capability of differentiating into numerous cell lineages and self-renewal are characteristics of stem cells (Thomson et al. 1998). Stem cells can be classified into totipotent, pluripotent, multipotent, oligopotent, and unipotent depending upon their location and the inherent ability of differentiation into multiple cells (Thomson et al. 1998; Mimeault et al. 2007; Takahashi et al. 2007). The human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) unlocked diverse practical opportunities for cell-based therapies and served as important ex-vivo models to investigate human hereditary ailments (Thomson et al. 1998; Mimeault et al. 2007; Takahashi et al. 2007).

Unlike other organs, the immunologically privileged status and convenience for treatment make the human eye a paradigm for cellular therapies. The stem cell-mediated treatments include ocular and nonocular adult stem cell therapies and pluripotent stem cell-based treatments. The adult ocular stem cell therapies include the treatments derived from lens epithelial progenitor/stem cells (Lin et al. 2016), ciliary pigment-epithelial stem cells (Ahmad et al. 2000; Tropepe et al. 2000), retinal pigment epithelial stem cells (Salero et al. 2012), and retinal Muller stem cells (Ooto et al. 2004; Reichenbach and Bringmann 2013). Different studies have documented the differentiation of iPSCs and hESCs into specified ocular cells, corneal epithelial cells (Hayashi et al. 2012), retinal pigment epithelial cells (Buchholz et al. 2009; Hirami et al. 2009), photoreceptors, and retinal ganglion cells (Osakada et al. 2008; Lamba et al. 2009; Eiraku et al. 2011; Homma et al. 2013; Zhong et al. 2014; Maekawa et al. 2016).

Corneal stromal stem cells, corneal epithelial stem cells, and corneal endothelial progenitor cells have been documented to exhibit self-renewal potential (Cotsarelis

et al. 1989; Hall and Watt 1989; Pinnamaneni and Funderburgh 2012). So far, a small number of studies have shown the isolation of presumptive progenitor cells from eye lens (Hanna and O'Brien 1961; Persons and Modak 1970; Rafferty and Rafferty 1981; Zhou et al. 2006; Yamamoto et al. 2008). Recently, Lin et al. isolated lens epithelial stem cells in mammals and have shown that endogenous lens epithelial stem cells can achieve lens regeneration in macaques, rabbits, and human infants having cataracts (Lin et al. 2016).

Adult ocular stem cells, including multipotent retinal progenitor cells present in ciliary pigment epithelium, have been documented to generate photoreceptors (Xu et al. 2007; Ballios et al. 2012; Clarke et al. 2012; Del Debbio et al. 2013). The partial regeneration ability of retinal pigment epithelial cells has been reported (Fischer 2005). Recently, a small population of retinal pigment epithelial cells has shown stem cell-like differentiation potential into several retinal cell types (Osakada et al. 2007; Lu et al. 2013).

In this chapter, we discuss the stem cell populations residing in different compartments of the eye with an overall focus on their potential in cell-based therapies. This is an update to the chapter titled stem cell for ocular therapies in DE Gruyter 2018 publication, Stem cells: from hype to real hope.

Cornea

The cornea, an avascular transparent tissue, is an outermost layer that covers the iris, pupil, and anterior chamber of the eye (Delmonte and Kim 2011). The cornea comprises three cellular layers, including endothelium, stroma, and epithelium, and two noncellular layers, i.e., Descemet's membrane and Bowman's layer (Delmonte and Kim 2011). As the outermost layer of the eye, the cornea serves as a barrier and provides an optical function and accounts for about two-third of the entire refractive potential of the human eye (Delmonte and Kim 2011).

Corneal Epithelium

The corneal epithelium is a stratiform, nonkeratinizing, and the outermost layer of the cornea (Cotsarelis et al. 1989; Dua et al. 2003). The stem cells present in the limbal lower region, also called limbal stem cells, exhibit self-repairing capability of the corneal epithelium (Cotsarelis et al. 1989; Hall and Watt 1989). The limbal stem cells give rise to two daughter cells, an oligopotent-limbal stem cell and the other cell form transient fast-dividing cells which eventually make terminally differentiated cells (Schermer et al. 1986; Tseng 1989b; Kruse and Tseng 1993; Kruse 1994; Zieske 1994; Pellegrini et al. 2009).

The limbal stem cell deficiency due to the loss of limbal stem cells leads to the malfunction of limbus barrier function, new vessel formation, and the loss of corneal transparency (Dua et al. 2000). The limbal stem cell deficiency could be caused by inherited stem cell-aplasia, ocular surface injury, idiopathic diseases, and stem cell

exhaustion (Hughes 1946; Tseng 1989a; Nishida et al. 1995; Dua et al. 2000; Ramaesh et al. 2005).

Corneal Stroma

The corneal stroma is a collagenous-connective tissue and accounts for 80–90% of the total thickness of the cornea (Patel et al. 2001, 2002). The keratocytes, proteoglycans, collagens, and glycoproteins are accountable for collagen formation in the stroma (Maurice 1957; Freegard 1997; Knupp et al. 2009).

Corneal stromal fibrosis (also known as stromal scarring) can occur as a consequence of surgery and/or infection that contributes to a major portion of corneal blindness worldwide (Shortt et al. 2010; Oliva et al. 2012). During stromal fibrosis, the keratocytes differentiate into fibroblasts that result in a disorganized extracellular matrix in the stroma (Cintron et al. 1973; Fini 1999; Funderburgh et al. 2001; Funderburgh et al. 2003). Corneal transplantation is the only available remedy for corneal blindness (Tan et al. 2012). However, the limited availability of cadaveric human stromal tissue compelled the requirement of alternative sources, i.e., the biosynthetic cornea (Lagali et al. 2011), human corneal stromal stem cells (hCSSCs), and the generation of stromal tissue using different cell types (Ren et al. 2008; Du et al. 2009; Du and Wu 2011; Arjamaa 2012; Espandar et al. 2012; Wu et al. 2012; Yoeruek et al. 2012; Wu et al. 2013; Basu et al. 2014; Boulze Pankert et al. 2014; Giasson et al. 2014; Katikireddy et al. 2014; Nakatsu et al. 2014). In a study, Funderburgh et al. showed specialized cells exhibiting mesenchymal stem cell characteristics from bovine corneal stroma (Funderburgh et al. 2005).

In a subsequent study, Du and coworkers isolated the keratocyte progenitor cells from the human corneal stroma and identified the expression of ABCG2, an adult stem cell marker in keratocyte progenitor stromal cells (Du et al. 2005). Several studies have reported the stromal cells demonstrating properties similar to mesenchymal stem cells (Amano et al. 2006; Yoshida et al. 2006; Polisetty et al. 2008; Branch et al. 2012; Garfias et al. 2012; Li et al. 2012). Du et al. injected hESCs in the Lumincan-null mice stroma which resulted in stromal-transparency (Du et al. 2009). Pinnamaneni and Funderburgh documented the potential of hESCs for the treatment of stromal-scarring (Pinnamaneni and Funderburgh 2012).

Corneal Endothelium

The corneal endothelium is a monolayer of hexagonal cells located on the posterior surface of the cornea and serves as a barrier against the uncontrolled flow of the aqueous humor into the cornea (Bonanno 2012). The corneal endothelium is essential for corneal transparency by mediating hydration through barrier and pump functions (Bonanno 2012). The corneal endothelial cell density is ~2500 cells/mm^2 in normal adult corneal endothelium (Tan et al. 2012). Surgical fumble and inherited corneal endothelial dystrophies are the leading causes responsible for

corneal endothelial cell loss and reduction in corneal endothelial cell density (Lorenzetti et al. 1967). The corneal endothelium function is strikingly affected below 500 cells/mm^2 corneal endothelial cell density resulting in corneal edema and loss of vision (Tan et al. 2012).

To date, several studies have been performed to recognize corneal endothelial-stem cells. In two independent studies, the researchers conducted a sphere-forming assay to identify human corneal endothelial stem cell-precursors (Yokoo et al. 2005; Amano et al. 2006). The analysis demonstrated no expression of stem cell-associated markers in precursor cells; however, the clonogenic potential, proliferative capacity, and the ability to form a hexagonal monolayer of cells may indicate that they are corneal endothelial progenitor cells (Yokoo et al. 2005; Amano et al. 2006). In addition, the sphere-forming assay revealed a higher propensity of sphere-forming in peripheral cells compared to centrally residing cells of the endothelium (Mimura et al. 2005; Yamagami et al. 2007). In another study, likely progenitor cells were recognized in the region among Schwalbe's line and peripheral endothelium (Bednarz et al. 1998; Whikehart et al. 2005; Mimura and Joyce 2006; He et al. 2012). Increased density and proliferative potential of corneal endothelial cells situated at the peripheral portion of the cornea in comparison to the middle portion further supported the identification of progenitor-like cells (Bednarz et al. 1998; Whikehart et al. 2005; Mimura and Joyce 2006; He et al. 2012).

In addition, multiple studies were executed to document the expression of stem cell-associated markers in stem/progenitor-like cells in human corneal endothelium (Hirata-Tominaga et al. 2013; Hara et al. 2014). Hirata-Tominaga et al. discovered the expression of a stem cell marker i.e., LGR5, in the peripheral cells of corneal endothelium (Hirata-Tominaga et al. 2013). The authors stated that LGR5 (+) corneal endothelial cells exhibit elevated proliferative potential compared to LGR5(−) cells. They further demonstrated that LGR5 expression is responsible for endothelial phenotype and also impedes endothelial-mesenchymal-transformation (Hirata-Tominaga et al. 2013). Based on the neural crest origin of human corneal endothelial cells, several studies employed neural crest-associated markers to separate and typify the likely progenitor-cells from human corneal endothelium (Hara et al. 2014; Katikireddy et al. 2016). However, the progenitor cells from the young cornea exhibited higher proliferative potency compared with the older cornea (Katikireddy et al. 2016).

In a recent study, the authors explored the neural crest origin of likely proenitor-cells separated from Fuchs endothelial corneal dystrophy and normal corneas (Katikireddy et al. 2016). These neural crest-originated progenitor cells revealed the nonexistence of senescence with an increase in passage number, sphere-forming potential, and elevated colony-making capability in contrast to the primary cells (Katikireddy et al. 2016).

Ali et al. reported differentiation of peripheral blood mononuclear cells originated, iPSCs to corneal endothelial cells which share a similar proteome profile with human corneal endothelium, and further demonstrated that cryopreservation does not affect the cardinal features of corneal endothelial cells (Ali et al. 2018b). The authors subsequently confirmed that iPSCs- and hESCs-derived corneal endothelial cells have comparable transcriptome profiles (Ali et al. 2018a). In a recent study, Ali

et al. demonstrated the efficacy of cryopreserved hESC-derived corneal endothelial cells to form a functional corneal endothelium on the denuded Descemet's membrane (Ali et al. 2021). The authors generated pluripotent stem cell-derived corneal endothelial cells under xeno-free conditions, characterized cryopreserved pluripotent stem cell-derived corneal endothelial cells using next-generation RNA sequencing, and successfully evaluated intracameral injection of cryopreserved corneal endothelial cells to form a functional corneal endothelium in rabbits and monkeys (Ali et al. 2021).

Conjunctiva

Conjunctiva is a mucous membrane lining the eyelids and provides protection and lubrication to the eye (Inatomi et al. 1996; Di Girolamo 2011). The conjunctiva can be distinguished into three portions: the bulbar that covers the eye surface, the forniceal that forms the junction between the bulbar and the palpebral conjunctivas, and the palpebral that makes the lining of the undersurface of eyelids (Inatomi et al. 1996; Di Girolamo 2011).

Several studies have identified putative conjunctival stem cells in the conjunctiva (Wei et al. 1995; Pellegrini et al. 1999; Wirtschafter et al. 1999; Chen et al. 2003); however, the precise location of conjunctival stem cells is yet not well defined. Nagasaki et al. reported the even dissemination of conjunctival stem cells in the conjunctiva in GFP-labeled mice (Nagasaki and Zhao 2005; Stewart et al. 2015). In another study, Pellegrini et al. reported uniform distribution of conjunctival stem cells in the fornices and bulbar conjunctiva (Pellegrini et al. 1999). In a recent study, Stewart et al. inferred the conjunctival stem cell location in medial canthal and the inferior forniceal conjunctiva based on colony-forming potential and expression-pattern of stem cell-associated markers (Stewart et al. 2015). Further, stem cell features, i.e., reduced cell cycling, and the elevated proliferation rate were used to characterize the putative stem cell niche in the conjunctiva of rabbits (Wei et al. 1993; Wei et al. 1995). These studies revealed forniceal conjunctiva exhibiting a higher number of cells displaying stem cell characteristics compared to bulbar and palpebral conjunctiva (Wei et al. 1993, 1995).

To date, conjunctival stem cell specific markers have not been identified; nevertheless, *ABCG2* and *p63* are considered to be the most reliable markers (Watanabe et al. 2004; de Paiva et al. 2005; Kawasaki et al. 2006). Budak et al. reported that the human bulbar region of the conjunctiva possesses *ABCG2* positive cells which exhibit features similar to epithelial stem cells (Budak et al. 2005). Likewise, Vascotto and Griffith documented the existence of *ABCG2* and *p63* positive cells in the bulbar portion of the conjunctival-epithelium (Vascotto and Griffith 2006).

Iris

The iris is a thin, annular structure that splits the space among the lens and cornea (Grierson et al. 2002). The iris is composed of four layers: the anterior border layer, the stroma, the dilator muscle layer, and the posterior epithelium double monolayers

of iris pigment epithelial cells on the posterior part of the iris (Freddo 1984; Grierson et al. 2002).

Different authors documented the potential of iris pigment epithelial cells as progenitor cells based on their growth in spheres and the expression of neural stem/progenitor cells (Arnhold et al. 2004; Seko et al. 2012). In two sovereign studies, the analysis showed that iris pigment epithelial cells can also be differentiated into glial and neuronal cell types (Arnhold et al. 2004; Seko et al. 2012). Based on their origin, the iris pigment epithelial cells can form a complete lens in adult newt (Eguchi 1988; Tsonis and Del Rio-Tsonis 2004). Similarly, Kosaka et al. showed that one day old chicken iris pigment epithelial cells can sustain a differentiated form and may give rise to retinal pigment epithelium and lentoid bodies (Kosaka et al. 1998). In another study, Sun et al. isolated chicken iris pigment epithelial cells exhibiting sphere-forming potential and expression of retinal progenitor markers in non-adherent culture (Sun et al. 2006). In addition, they showed that iris pigment epithelial cells derived from postnatal chicken can proliferate similar to neural progenitor/stem cells with the ability to regenerate several cell types, such as retina-associated glia, neurons, and the lens (Sun et al. 2006).

Ciliary Body

The ciliary body is comprised of epithelium and muscle that is mainly involved in the production of aqueous humor (Napier and Kidson 2007). The ciliary body also regulates intraocular pressure, aqueous flow, and the maintenance of the immune-privileged status of the anterior chamber of the eye.

Multiple studies have demonstrated the presence of multipotent stem cells in the ciliary pigment epithelium isolated from adult mice, rats, rabbits, porcine, and humans (Ahmad et al. 2000; Tropepe et al. 2000; Inoue et al. 2005; Abdouh and Bernier 2006; MacNeil et al. 2007; Moe et al. 2009). In three independent studies, the analyses revealed about 1 in 100 and 1 in 500 ciliary body cells have proliferative capability isolated from adult mice and rats, respectively (Tropepe et al. 2000; Das et al. 2004; Kohno et al. 2006). Further, it has been reported that ciliary pigment epithelium-derived progenitor cells can be identified and isolated using neural retinal and/or progenitor associated markers (Xu et al. 2007; Moe et al. 2009). Xu et al. documented isolation of progenitors-like cells from the ciliary body of adult rats, mice, and human cadaver eyes (Xu et al. 2007) and have shown the expression of retinal/neuronal markers and neurospheres formation by ex vivo culturing of ciliary pigment epithelium-derived progenitor cells (Xu et al. 2007).

Trabecular Meshwork

The trabecular meshwork is a triangular-shaped tissue that lies in between the iris and cornea and regulates the outflow of aqueous humor fluid in the eye (Buller et al. 1990). The features of trabecular meshwork cells include the secretion of specific

enzymes, extracellular matrix, and phagocytosis in the aqueous humor (Buller et al. 1990; Stamer et al. 1995).

The attenuation in trabecular meshwork-cellularity was observed with the progression of age and glaucoma and linked with elevated outflow resistance and increased intraocular pressure (Alvarado et al. 1981, 1984; He et al. 2008). Multiple studies have documented a subpopulation of cells that express mesenchymal cell-specific markers, escape replicative senescence in human primary trabecular meshwork cells, and the potential to differentiate into various lineages by characterizing trabecular meshwork cells (Du et al. 2012; Tay et al. 2012). Recently, Du et al. reported phagocytic activity of multipotent stem cells from human trabecular meshwork tissue (Du et al. 2012). Further, multipotent stem cells isolated from trabecular meshwork revealed the expression of pluripotent stem cell- and mesenchymal stem cell-markers examined by gene expression analysis (Du et al. 2012, 2013). These cells also demonstrated the expression of trabecular meshwork-associated markers including *MGP, AQP1, CHI3L1,* and *TIMP3* (Du et al. 2012, 2013). Similarly, multiple studies reported stem cell-like cells in primary trabecular meshwork cultures which can form free-floating neurospheres (Vittitow and Borris 2004; Gonzalez et al. 2006; McGowan et al. 2007; Xu et al. 2007; Kelley et al. 2009).

Lens

The lens is structurally transparent tissue without vessels which is mainly responsible to focus light on the retina (McAvoy et al. 1999; Cvekl and Ashery-Padan 2014). The lens originates from the head-ectoderm which forms the lens placode. The lens placode further turns inward along with the optic vesicle to constitute the lens pit, and the optic cup, respectively (McAvoy et al. 1999; Cvekl and Ashery-Padan 2014). Next, the lens pit detaches from the ectoderm to create the lens vesicle which generates anterior and posterior single layers of cells (McAvoy et al. 1999; Cvekl and Ashery-Padan 2014). The lens develops quickly during late-embryonic and early post birth stages as the epithelial cells differentiate into secondary fiber cells (McAvoy et al. 1999; Cvekl and Ashery-Padan 2014). The closely packed lens fiber-cells, the maximum quantity of crystallin proteins, and the organelle loss during the differentiation process lessen light scattering and offer the refractive index essential for transparency and focusing (Michael et al. 2003; Bloemendal et al. 2004).

The quest of presumptive progenitor cells identification in the eye lens has been investigated in multiple studies (Hanna and O'Brien 1961; Persons and Modak 1970; Rafferty and Rafferty 1981; Zhou et al. 2006; Yamamoto et al. 2008). Overall, these investigations focused on the identification of slow and fast cycling-cells employing DNA-tagging methods in chick, rat, and mouse lenses (Hanna and O'Brien 1961; Persons and Modak 1970; Rafferty and Rafferty 1981; Zhou et al. 2006; Yamamoto et al. 2008). The epithelial cells situated at the peripheral zone of lens epithelium in the transition region exhibit increased proliferative potential. In contrast, the central portion of the lens demonstrates mitotically inactive epithelial

cells (Hanna and O'Brien 1961; Persons and Modak 1970; Rafferty and Rafferty 1981; Zhou et al. 2006; Yamamoto et al. 2008). It is well documented that lentoid bodies and lens progenitor cells can be generated from iPSCs and hESCs (O'Connor and McAvoy 2007; Yang et al. 2010; Qiu et al. 2012; Li et al. 2016; Fu et al. 2017; Ali et al. 2019, 2020). We previously showed that lentoid bodies can be generated from hESCs and peripheral blood mononuclear cells.

Originally, both hESCs and iPSCs shared similar morphological characteristics and expression pattern. We generated lentoid bodies using the "fried egg" method (Ali et al. 2019). Both hESCs and iPSCs were cultured on Matrigel-coated plates in mTeSR1 medium. The fried-egg like structures were observed on day 8 during the differentiation procedure. The phase-contrast microscopy of hESC- and iPSC-derived lentoid bodies revealed lens-like morphological appearance on differentiation day 25 (Fig. 1).

The lentoid bodies were harvested at differentiation day 25 and subjected to next-generation RNA sequencing (RNA-Seq) analysis. The mapped reads represented 263.42x and 238.01x sequence coverage for hESC- and iPSC-derived lentoid bodies, respectively, for ~70 Mb human transcriptome (Ali et al. 2019). The datasets revealed the expression ($\geq$ 0.659 RPKM) of 13,975 and 14,003 genes in hESC- and iPSC-derived lentoid bodies, respectively (Ali et al. 2019). A comparative analysis of these data suggested similar transcriptomes of hESC- and iPSC-derived lentoid bodies (Ali et al. 2019).

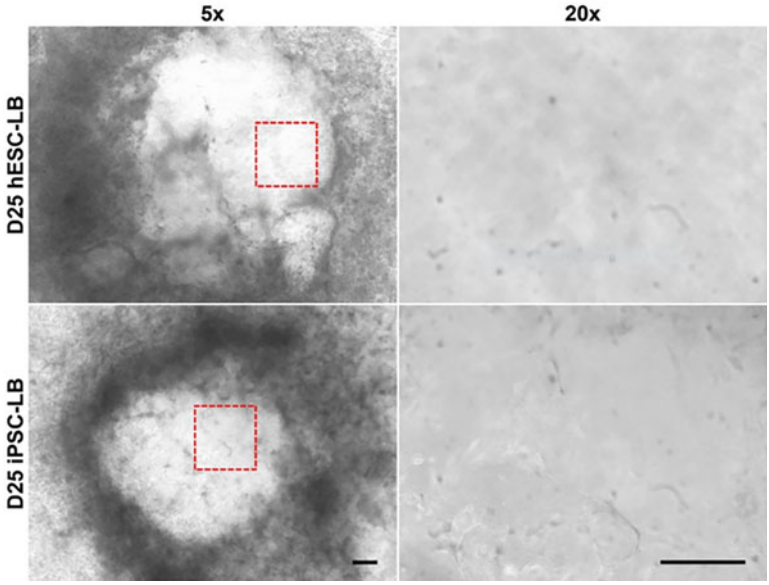

Fig. 1 Generation of lentoid bodies from H9 human embryonic stem cells (hESCs) and peripheral blood mononuclear cell (PBMC)-originated, induced pluripotent stem cells (iPSCs). The red dotted square indicates a lens-like transparent structure on differentiation day 25. The images are of 5x and 20x magnifications, and the scale bars represent 100 μm

Retina

The retina is a complicated structure, comprised of neural cell layers (ganglion cells, bipolar cells, and photoreceptor cells) and retinal pigment epithelium (Raymond and Hitchcock 1997). The human retina has reduced repair potential. Multiple retinal disorders including age-related macular degeneration (AMD), retinitis pigmentosa (RP), diabetic retinopathy, glaucoma, and Leber congenital amaurosis (LCA) have been reported to cause retinal damage and eventually resulting in vision loss (Alonso-Alonso and Srivastava 2015).

The human retina has shown restricted renewal potential; however, the neural retina can be regenerated in lower vertebrates (Raymond and Hitchcock 1997; Reh and Levine 1998). In amphibians and fish, retinal injury can prompt the proliferation of retinal pigment epithelial cells (Mitashov 1997). Fischer and Reh documented that the postnatal chicken retina exhibits the regeneration potential to generate neurons (Fischer and Reh 2001). Following injury, Muller glial cells enter the cell cycle, dedifferentiate, attain progenitor-like properties, and generate new neurons and glia (Fischer and Reh 2001). The retina of adult birds (Goldman and Nottebohm 1983) and mammals (Tropepe et al. 2000; Fischer and Reh 2001) have demonstrated the presence of multipotent stem cells.

Retinal Pigment Epithelium

The retinal pigment epithelium originates from the outer layer of the optic cup (Rapaport et al. 1995). Multiple reports have shown the proliferation proficiency of retinal pigment epithelium harvested from the adult and fetal human retina (Hu and Bok 2001; Maminishkis et al. 2006; Blenkinsop et al. 2012). Salero et al. explored a retinal pigment epithelial cell subpopulation that can be prompted to a stem cell-state presenting self-renewing and multipotent competencies (Salero et al. 2012). Blenkinsop et al. reported that retinal pigment epithelial stem cells can divide efficiently and retain the capability to differentiate into an exceedingly polarized retinal pigment epithelium monolayer (Blenkinsop et al. 2012).

Biswas et al. recently reported detection and validation of the underlying cause of progressive retinal degeneration (Biswas et al. 2021). Whole-genome sequencing identified a novel intron 8 donor splice site variant (c.1296 + 1G > A) and a novel exonic deletion encompassing the Mer tyrosine kinase proto-oncogene (*MERTK*), which is highly expressed in the retinal pigment epithelium (Biswas et al. 2021). To evaluate the impact of these novel variants, the authors reprogrammed peripheral blood mononuclear cells of the patient and parents of the patient to human iPSC lines, which were differentiated to retinal pigment epithelium (Biswas et al. 2021). The analysis of human iPSC–derived retinal pigment epithelium revealed the absence of both *MERTK* transcript and its respective protein as well as abnormal phagocytosis when compared with the parental human iPSC–retinal pigment epithelium (Biswas et al. 2021). Importantly, this approach using a human iPSC-derived retinal pigment epithelium model to establish the functional impact of disease-causing mutations to establish the potential mechanism underlying retinal pathology advocates for the use of patient-specific iPSCs. Characterization of iPSC-derived

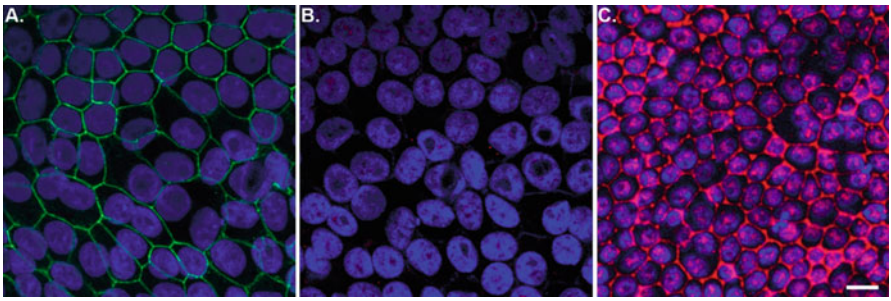

Fig. 2 Characterization of human induced pluripotent stem cell (iPSC)-derived retinal pigment-epithelial cells. Human iPSC-derived, retinal pigment epithelium is examined for the expression of multiple retinal pigment-epithelium markers including (**a**) Zona occludens-1 (ZO1), (**b**) the helix-loop-helix leucine zipper transcription factor MiTF, stained red, and (**c**) BEST1 localized to the basal side of human iPSC–retinal pigment epithelium which are shown. Scale bar: 10 μm

retinal pigment epithelial cells was confirmed by examining the expression of retinal pigment epithelium-associated markers including zona occludens-1 (ZO1), the helix-loop-helix leucine zipper transcription factor MiTF, and BEST1 (Fig. 2).

Retinal Muller Cells

Multipotent retinal progenitor cells give rise to Muller glial cells (Martin and Grünert 1992; Strettoi and Masland 1995), which constitute ~4–5% of total cells of the mouse retina (Young 1985; Jeon et al. 1998). It is well documented that retinal Muller cells can differentiate into various retinal cell types (Bernardos et al. 2007; Jadhav et al. 2009). Multiple studies reported the limited regeneration ability of Muller cells in chicken (Fischer and Reh 2001), rats (Reyes-Aguirre et al. 2013; Ferraro et al. 2015), mice (Ooto et al. 2004; Karl et al. 2008), and humans (Lawrence et al. 2007). It has been reported that Muller cells can reenter the cell cycle and redevelop the ganglion cells and inner nuclear layer in the injured retina of zebrafish (Raymond et al. 2006; Fimbel et al. 2007). Muller glia cells have also shown the regeneration potential into different types of neurons following N-methyl-d-aspartate (NMDA) injury to the retina in rodents (Ooto et al. 2004). Wan et al. documented photoreceptor repair in NMDA-induced retinal photoceptor-degenerated adult rats using Muller glial cells (Wan et al. 2008). Further studies have demonstrated the differentiation capability of Muller glia into ganglion cells under defined conditions (Zhao et al. 2014; Song et al. 2015, 2016).

Summary

In summary, we have provided a comprehensive view of adult stem cell populations residing in various compartments of the eye with an overall focus on their functional applications in cell-based therapies. Multiple studies have characterized adult stem

cells in different human body tissues including the eye and highlighted the crucial role of adult stem cells in maintaining natural homeostasis. Stem cells have distinct properties which permit them to regulate cell replacement during homeostasis and tissue repair. During homeostasis and after injury, the stem cells divide to generate daughter-transient amplifying cells which proliferate, migrate, and differentiate to replace the missing cells. However, this cannot happen when the stem cell population is depleted as a result of some diseased condition or trauma. The stem cell activity in any system requires to be strictly regulated for keeping the tissue in equilibrium. In this regard, the niche or tissue microenvironment, where stem cells exist, plays a crucial role in regulating stem cell fate decisions. Within these specified microenvironments, multiple signals are delivered, which guarantee correct stem cell activity. However, the exact cellular and molecular mechanisms by which the niche controls stem cell behavior remain elusive. It is important to note that stem cells are profound to the microenvironment they are exposed to, and irrelevant microenvironmental signals may lead to their depletion or impaired function. Therefore, further studies are required to conclude how stem cells are regulated by the niche and how niche components alter during infection or injury which ultimately will improve therapeutic strategies for the treatment of multiple ocular dysfunctions.

Cross-References

► The Potential of Stem Cells in Ocular Treatments

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The Potential of Stem Cells in Ocular Treatments

21

S. Amer Riazuddin, Shahid Y. Khan, and Muhammad Ali

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Abstract

Although conventional treatment strategies of drug administration and surgical intervention have been successful in protecting vision loss, however these efforts have been unable to discover a comprehensive approach to prevent the progression of disease in many ocular disorders. In this context, stem cell-based therapies provide an alternative approach to prevent vision loss in instances where conventional treatments have failed. The immune-privileged status and easy accessibility of different tissue of the eye offers several advantages for stem-cell-based therapeutic intervention. The pluripotent stem cells have been used to generate multiple eye cell types including corneal epithelial- and endothelial-cells, retinal pigment-epithelial cells, photoreceptors, retinal ganglion cells, and lens, conjunctival, limbal, and trabecular meshwork cells. Multiple pluripotent stem cell-based clinical trials for the treatment of a wide range of eye diseases are in progress.

S. A. Riazuddin (✉) · S. Y. Khan · M. Ali
The Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA
e-mail: sriazud1@jhmi.edu; skhan59@jhmi.edu; mriaz1@jhmi.edu

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In this chapter, we review the potential of pluripotent stem cell-derived cells of multiple eye tissues to prevent vision loss by replacing the degenerative cells.

Keywords

Embryonic stem cells · Eye · Induced pluripotent stem cells · Ocular · Preclinical · Retinal · Stem cells

Abbreviations

AMD	Age-related macular degeneration
BL	Bowman's layer
CE	Corneal endothelium
DM	Descemet's membrane
ESCs	Embryonic stem cells
iPSCs	Induced pluripotent stem cells
RGCs	Retinal ganglion cells
RP	Retinitis pigmentosa
SEAM	Self-formed ectodermal autonomous multizone

Introduction

Stem cell-based regenerative medicine is an emerging field where stem cells or their derivatives have been used to regenerate and restore the physiological functioning of several ocular tissues in preclinical models and human patients (Streilein et al. 2002; Jones et al. 2017). The mammalian eye has an intricate structure and harbors various advantages for stem cell-based therapies. Retinal degenerative diseases, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), are characterized by the early loss of photoreceptors or retinal pigment-epithelial cells (de Jong 2006; Ferrari et al. 2011), while glaucoma is characterized by retinal ganglion cell (RGC) degeneration (Davis et al. 2016a). These clinical conditions lead to the loss of photoreceptors over time, resulting in permanent blindness (Berson 1993; Quigley 1993; de Jong 2006).

Over the past two decades, many ocular cell types have been generated through differentiation of embryonic stem cells (ESCs) and, more recently, using induced pluripotent stem cells (iPSCs) including corneal epithelial cells (Zhu et al. 2018), retinal pigment-epithelial cells (Buchholz et al. 2009; Hirami et al. 2009), photoreceptors, and retinal ganglion cells (Osakada et al. 2008; Lamba et al. 2009; Eiraku et al. 2011; Homma et al. 2013; Zhong et al. 2014; Maekawa et al. 2016). Importantly, the generation of these cells has propelled their use in therapeutic applications to cure degenerative ocular diseases. Among these, limbal stem cells and corneal endothelial cells have been examined for their use in treating clinical conditions that involve degeneration of the corneal epithelium (Daniels et al. 2007; O'Sullivan and Clynes 2007) and corneal endothelium, respectively (Ali et al. 2021). Human pluripotent stem cell-derived trabecular meshwork cells (Du et al. 2012; Zhu et al.

2016, 2017) and RGCs (Kador et al. 2013; Sluch et al. 2015; Venugopalan et al. 2016) have been investigated for treating glaucoma.

Moreover, amacrine, retinal Muller cells, and retinal pigment epithelium derived from stem cells have been examined for the treatment of retinal diseases including RP and AMD (Dyer and Cepko 2000; Fischer and Reh 2001; Fausett and Goldman 2006; Bernardos et al. 2007; Lenkowski and Raymond 2014; Chen et al. 2015).

In this chapter, we discuss advances in stem cell-based therapeutic routes for the restoration of the physiological functioning of ocular tissues. This is an update to the chapter titled "Stem cells in ophthalmology" in DE Gruyter 2018 publication, Stem cells: from hype to real hope.

Cornea

Cornea is the outermost tissue of the eye consisting of three cellular layers (epithelium, stroma, and endothelium) and two acellular layers including Descemet's membrane and Bowman's layer (also known as Bowman's membrane) (Fig. 1). The cornea is responsible for two-thirds of the refractive power of the eye, and it provides a protective barrier against infection and debris entering the eye (Delmonte and Kim 2011).

Corneal Epithelium

The corneal epithelium is comprised of polygonal superficial cells, middle wing cells, and small basal cells (Delmonte and Kim 2011). The epithelium stem cells, also called limbal stem cells, are located in a limbal basal layer, the region between the conjunctiva and cornea (Delmonte and Kim 2011). The physiological damage

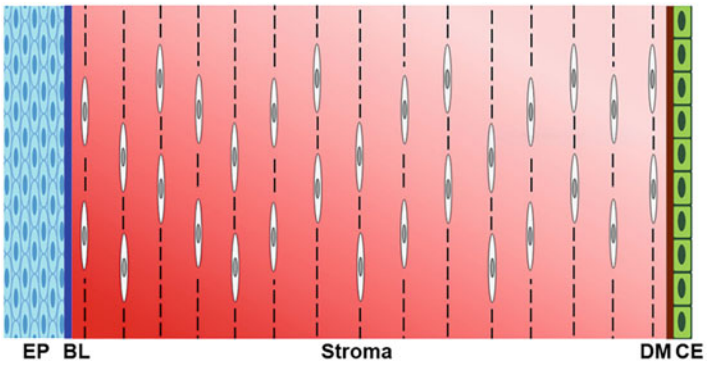

Fig. 1 A schematic of the three cellular layers, the epithelium (EP), stroma, and corneal endothelium (CE), and two acellular layers, Bowman's layer (BL) and Descemet's membrane (DM) of the cornea

and/or deterioration of the limbal epithelial cells leads to the limbal-stem-cell-deficiency (Dua et al. 2003).

Different studies have documented the capability of limbal stem cells to treat limbal-stem-cell-defeciency by substituting the disintegrated corneal epithelium (Kenyon and Tseng 1989; Ilari and Daya 2002; Gomes et al. 2003; Utheim 2013). It is well documented that transplantation of autologous cultured limbal stem cells can repair human limbal-stem-cell-deficiency (Pellegrini et al. 1997). Both allogeneic and autologous transplantation of limbal stem cells have been reported (Kenyon and Tseng 1989); nevertheless, these are hindered by posttransplantation complications and donor shortage (Shimazaki et al. 2000; Ilari and Daya 2002; Samson et al. 2002; Gomes et al. 2003).

The treatment of unilateral limbal-stem-cell-deficiency patients using autologous limbal stem cells transplantation has shown promising results. However, it is worth noting that other source(s) of limbal stem cells will be needed for the treatment of patients with bilateral limbal-stem-cell-deficiency. To circumvent these hurdles, human autologous buccal-epithelial stem cells originated from epithelial cell sheets were employed to treat bilateral limbal-stem-cell-deficiency patients (Priya et al. 2011). Further, Homma et al. examined the potential of ESC-derived epithelial progenitors to treat impaired cornea (Homma et al. 2004). Hayashi et al. differentiated human iPSCs into corneal epithelial-like cells (Hayashi et al. 2012). Next, Hayashi et al. generated self-formed ectodermal autonomous multizone (SEAM) ocular cells from iPSCs and demonstrated that cells from the SEAM can be separated and expanded to construct a corneal epithelium. The reconstructed corneal epithelium successfully mended the corneal blindness in an animal model (Hayashi et al. 2016).

Corneal Stroma

The stroma is comprised of collagens, glycoproteins, keratocytes, and proteoglycans, which play a crucial role in collagen formation (Maurice 1957; Freegard 1997; Knupp et al. 2009). Stromal fibrosis, as a result of infection, surgery, or ocular trauma, can lead to opacification, which is a substantial contributing factor to corneal blindness globally (Shortt et al. 2010; Oliva et al. 2012). To date, the only therapeutic option for the treatment of corneal stromal disease is deep anterior lamellar keratoplasty (Tan et al. 2012). However, this approach has restrictions such as lack of donor corneas and immune rejection (Tan et al. 2012).

Further, immortalized human corneal cells have been employed to generate functional human corneal equivalent. Buznyk et al. reported successful rehabilitation of visual acuity in two out of three patients by transplanting bioengineered corneal grafts (Buznyk et al. 2015), while Basu et al. documented the repair of injured stroma in mice by implanting human limbal biopsy-originated stromal cells (Basu et al. 2014). Furthermore, many studies have generated stromal keratocytes by differentiation of pluripotent stem cells, adult dental pulp stem cells, and mesenchymal stem cells (Syed-Picard et al. 2015; Zhang et al. 2015; Hertsenberg and Funderburgh 2016).

Stem Cell-Based Treatment for Corneal Edema

The corneal endothelium, the innermost layer of the cornea comprising of hexagonal/polygonal cells, is essential to retain corneal transparency by maintaining hydration through the pump and the barrier functions (Bonanno 2012). Corneal hydration is maintained primarily by the balance between the aqueous humor flow into stroma through the corneal endothelium and pumping out the fluid from the stroma (Nishida et al. 2010; Dawson et al. 2011). The accumulation of fluid in the stroma as a result of disruption of this balance may cause corneal opacity with reduced transparency. Tight junctions regulate the flow of aqueous humor through the corneal endothelium into the stroma, and sodium- and potassium (Na^+- and K^+)-dependent ATPase in the basolateral membrane is accountable for the pump function of the corneal endothelium (Dawson et al. 2011). The corneal endothelial cell density is approximately 2500 cells per square millimeter (cells/mm^2) in adult corneal endothelium, and the functioning of the corneal endothelium is significantly affected below a density of 500 cells/mm^2 resulting in corneal edema that eventually leads to vision loss (Tan et al. 2012).

Surgical trauma and corneal endothelial dystrophies are mainly responsible for corneal endothelial cell loss and a reduction in corneal endothelial cell density (Lorenzetti et al. 1967). Keratoplasty has been successful in visual restoration; however, the complexity of the procedure, the shortage of donor tissue, and graft rejection continue to impede the efforts to reduce corneal-blindness globally (Tan et al. 2012). The eye banking and worldwide estimation of corneal transplantation report the considerable shortage of corneal graft tissue, with only 1 cornea available for 70 needed (Gain et al. 2016), which compels for the development of alternative therapies.

Multiple alternate sources including ESCs, neural crest cells, adipose-derived stem cells, skin-derived precursors, and corneal stromal stem cells have been explored for corneal endothelial cell generation. Many research groups have shown that corneal endothelial cells can be generated by the differentiation of pluripotent stem cells (Fukuta et al. 2014; Zhang et al. 2014; McCabe et al. 2015; Song et al. 2016; Zhao and Afshari 2016). Fukuta et al. generated corneal endothelial cells from pluripotent stem cells using chemically defined conditions (Fukuta et al. 2014). Further, Zhang et al. reported restoration of corneal clarity in rabbits by transplanting corneal endothelium-like sheets (Zhang et al. 2014).

Intracameral injection of cultured corneal endothelial cells has been shown to restore corneal endothelium function in corneal endothelial dysfunction rabbit and monkey models (Okumura et al. 2016). A preclinical study in a primate model revealed corneal endothelium regeneration after the injection of human corneal endothelial cells or cultured monkey corneal endothelial cells, along with a Rho kinase inhibitor, into the anterior chamber of the primates' eye (Okumura et al. 2016). Many groups have reported the use of Rho kinase inhibitor to augment the adhesion of cultured corneal endothelial cells (Okumura et al. 2009; Peh et al. 2015).

Recently, Kinoshita et al. reported a revolutionary study in corneal transplantation and successfully treated the human subjects suffering from bullous

keratopathy (BK) by injecting cultured human corneal endothelial cells along with a Rho kinase inhibitor (Y-27632) into the anterior chamber of the eye (Kinoshita et al. 2018). Pan et al. reported a stepwise strategy to generate corneal endothelial cells from mice fibroblasts under chemically defined conditions using small molecules and successfully reversed the corneal opacity by the transplantation of corneal endothelial cells into a rabbit model with BK (Pan et al. 2021). Shen and the co-authors reported the generation of corneal endothelial cell-like cells derived from human skin-derived precursors, and subsequent injection of corneal endothelial cell-like cells revealed excellent therapeutic outcomes in rabbit and monkey corneal endothelial dysfunction models (Shen et al. 2021). In a recent study, Ali et al. demonstrated the efficacy of cryopreserved human embryonic stem cell (hESC)-derived corneal endothelial cells to form a functional corneal endothelium on the denuded Descemet's membrane (Ali et al. 2021). The authors first demonstrated through comparative gene expression analysis that cryopreservation of hESC-derived corneal endothelial cells for more than 5 weeks in liquid nitrogen does not affect their cardinal features.

In parallel, the authors developed animal models mimicking corneal endothelium edema by removing the central corneal endothelium. The cryopreserved hESC-derived corneal endothelial cells were injected into the anterior chamber of the corneal endothelium dysfunction animal (i.e., rabbit and monkey) models. As reported by Ali and colleagues, the transparency of the cornea reduced in the initial days after removal of central corneal endothelium; however, the transparency improved after the intracameral injection of hESC-derived corneal endothelial cells, and the transparency of the injected corneas became comparable to the uninjected control eyes in all animal models within 3 weeks postinjection (Fig. 2). Importantly, necropsy examination of these preclinical animal models confirmed no remarkable change in multiple tissues examined for tumorigenesis, alleviating the concerns of teratoma formation associated with the use of pluripotent stem cells and/or pluripotent stem cell-derivatives.

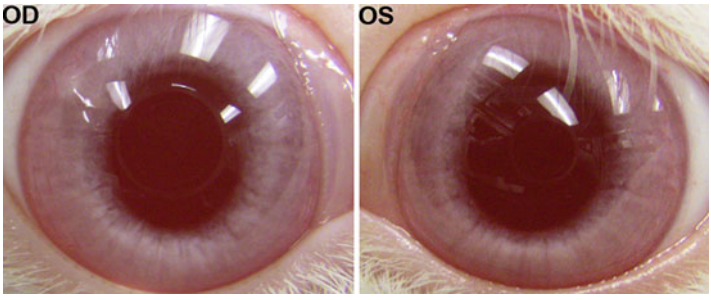

Fig. 2 Injection of human pluripotent stem cell-derived corneal endothelial cells into anterior chamber results in the generation of a functional monolayer of the corneal endothelium within 3 weeks. Importantly, the transparency of the injected right eye (OD) is comparable to the untreated left eye (OS). OD: oculus dextrus (right eye); OS: oculus sinister (left eye)

Trabecular Meshwork

The trabecular meshwork is critical for the drainage of the aqueous humor in the eye (Buller et al. 1990), and a reduction in trabecular meshwork cellularity (observed in case of glaucoma, and normal aging) links with high intraocular pressure (Alvarado et al. 1981, 1984). The general approach of treatments for glaucoma includes a drug-dependent decrease of aqueous humor formation and the surgical improvement of outflow to maintain normal intraocular pressure (Serle 1994; Kaufman and Rasmussen 2012). Stem cell-based therapy has remarkable potential for the repair of trabecular meshwork cellularity in aging and glaucoma patients (Pearson and Martin 2015). Alexander and Grierson were the first to report successful rehabilitation of trabecular meshwork function employing laser trabeculoplasty technique (Alexander and Grierson 1989). Laser trabeculoplasty treatment caused elevated trabecular meshwork cell division which is suggestive of the existence of "likely stem cells" in the anterior nonfiltering portion of the trabecular meshwork (Acott et al. 1989; Dueker et al. 1990). Du et al. examined the therapeutic efficacy of stem cell-like cells from human trabecular meshwork by transplanting them into mouse-trabecular meshwork tissue (Du et al. 2013). Differentiation of human embryoid bodies-derived iPSCs into trabecular meshwork cells has been reported by Abu-Hassan et al. (2015). In another study, mesenchymal stem cells were employed to regenerate the trabecular meshwork in laser-mediated glaucoma animal models (Manuguerra-Gagné et al. 2013). Next, Ding and coworkers employed primary human trabecular meshwork cell preconditioned media for the generation of trabecular meshwork cells from mouse iPSCs (Ding et al. 2014). Moreover, Zhu et al. evaluated the potential of iPSC-derived trabecular meshwork cells in a glaucoma mouse model (Zhu et al. 2016, 2017). It is well documented that iPSC-derived trabecular meshwork cells helped in decreasing intraocular pressure, repairing aqueous humor drainage, preventing retinal ganglion cell loss, and improving the proliferation of endogenous trabecular meshwork cells (Zhu et al. 2016, 2017).

Ocular Lens

Lens cloudiness or cataracts including age-linked and congenital cataracts are the primary cause of vision loss globally (Stevens et al. 2013). The most effective strategy for cataract treatment is the replacement of the cloudy lens with an artificial intraocular lens (Visser et al. 2013). Cataract surgical procedure is very successful in visual rehabilitation, however, linked with postsurgical hitches including posterior-capsule-opacification (Wormstone 2002; Lois et al. 2005). Further, the role of stem cells has been studied in lens regeneration (Tsonis and Del Rio-Tsonis 2004; Barbosa-Sabanero et al. 2012). It is well documented that in rabbits, the residual lens progenitor/epithelial stem cells give rise to a partial generation of lens fiber cells following removal of the eye lens (Gwon et al. 1990; Gwon 2006). Recently, Lin et al. documented an innovative approach for the elimination of cataractous lens by retaining resident lens-epithelial-cells and attained a functional restoration of the lens

in macaques, rabbits, and in human infants (Lin et al. 2016). O'Connor and McAvoy demonstrated that rat limbal epithelial cell monolayers can form functional lens-like structures ex vivo (O'Connor and McAvoy 2007). They noticed that regenerated rat lenses were meticulously similar to those of neonatal rat lenses and extended culturing of these regenerated rat lenses exhibited cataractogenesis (O'Connor and McAvoy 2007). Several groups have described that iPSCs and hESCs can form lens progenitor cells as well as lentoid bodies (Yang et al. 2010a; Qiu et al. 2012; Li et al. 2016; Fu et al. 2017b). Yang et al. generated lentoid bodies employing hESCs under chemically defined conditions (Yang et al. 2010b). The lentoid bodies produced by Yang et al. showed lens-associated makers; however, they do not exhibit optical properties (Yang et al. 2010b). Recently, Fu et al. developed human urinary cell, originated iPSC-derived lentoid bodies adopting the fried-egg approach (Fu et al. 2017a). These lentoid bodies demonstrated lens-associated markers as well as transparency similar to the human lens (Fu et al. 2017a). Moreover, Murphy et al. documented the human pluripotent stem cell-derived microlenses (Murphy et al. 2018). More recently, Ali et al. produced lentoid bodies from human iPSCs and hESCs and performed characterization using next-generation RNA sequencing (RNA-Seq) and TMT-based proteome profiling (Ali et al. 2019, 2020).

The Retina

Pluripotent stem cells have unlimited self-renewal capability, and pluripotent stem cell-derived retinal cells can be an ideal source to repair the degenerating retina. Human iPSCs and hESCs have been used for the generation of retinal photoreceptor cells and retinal pigment epithelium. To this end, several investigations have examined the therapeutic efficacy of adult progenitor/stem cells to treat retinal deteriorating diseases.

Salero et al. showed that the retinal pigment-epithelial stem cells (lacking the retinal pigment epithelial-associated markers) can proliferate and generate cobblestone-like monolayers (Salero et al. 2012). Blenkinsop et al. published a novel culture approach and proved that adult human eye-originated, retinal pigment epithelial cells can generate highly purified retinal pigment-epithelial cultures that demonstrate native retinal pigment epithelial-like properties (Blenkinsop et al. 2015). Moreover, Davis et al. documented that subretinal transplantation of retinal pigment-epithelial stem cell-derived retinal pigment-epithelial cells in rats inhibits loss of photoreceptors (Davis et al. 2016b, 2017). Further, Davis et al. reported that subretinal transplantation of retinal pigment-epithelial stem cell-derived retinal pigment-epithelial cells prevents vision loss in the retinal pigment-epithelial cell dysfunction rat model (Davis et al. 2017).

Mitashov et al. reported that Muller glial cells reveal characteristics similar to stem cells and contribute to the restoration of the damaged retinas in amphibians (Mitashov 1996). Additionally, Jayaram et al. showed photoreceptor cells, derived from Muller cells, when injected in the retinas of 3-week-old P23H-rats can

incorporate into the outer nuclear layer of deteriorated retinas, which results in improvement of rod photoreceptor function (Jayaram et al. 2014).

Several studies have documented the use of hESCs for the generation of neural retinal progenitor cells and functional retinal pigment-epithelial cells (Klimanskaya et al. 2004; Lamba et al. 2006; Lund et al. 2006; Osakada et al. 2008; Hirami et al. 2009; Idelson et al. 2009). It is well documented that three-dimensional organoid similar to the optic cup and the neural retina can be generated from mouse-pluripotent stem cells (Eiraku et al. 2011; Nakano et al. 2012; Zhong et al. 2014). Recently, DiStefano et al. generated retinal organoids employing rotating wall vessel-bioreactors from pluripotent stem cells (DiStefano et al. 2018). Gonzalez-Cordero et al. examined the integration-efficacy of rod precursor cells into the deteriorated retinas of adult mice (Gonzalez-Cordero et al. 2013). In another study, the authors transplanted three-dimensional retinal sheets derived from mouse iPSCs or ESCs in a retinal deterioration model in which the outer nuclear layer was absent (Assawachananont et al. 2014). In two different studies, the intraocular transplantation of neural precursor cells derived from hESCs and donor cells from developing retinas in the subretinal microenvironment presented integration and differentiation into various cell types (Banin et al. 2006; MacLaren et al. 2006).

It is worth noting that iPSC-based treatments showed a promising method for the repair of degenerative retinal diseases. iPSCs reveal a better option in contrast to hESCs because of the patient-specific method: utilizing the identical genetic background, decreasing the chances of immune rejection while also eliminating the numerous ethical concerns linked with ESCs. Moreover, genome-editing techniques such as transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) have been employed to correct patient-specific iPSCs which can be used for the repair of retinal deteriorating diseases (Hung et al. 2016; Chuang et al. 2017; Peng et al. 2017). Bassuk et al. reported editing of patient-specific iPSCs to correct a mutation present in an RP-GTPase regulator gene using CRISPR/Cas9 (Bassuk et al. 2016).

Several groups have reported that hESC- and iPSC-derived retinal pigment-epithelial cells exhibit comparable gene expression profiles and phenotypic characteristics (Buchholz et al. 2009; Meyer et al. 2009; Osakada et al. 2009). Additionally, many research groups have successfully generated neural retinal progenitor cells, such as photoreceptor cells from human iPSCs (Meyer et al. 2009; Osakada et al. 2009; Lamba et al. 2010; Mellough et al. 2012; Zhong et al. 2014). Multiple studies documented enhanced optical function following transplantation of iPSC-derived retinal pigment-epithelial cells in various RP-mouse models (Lopez et al. 1989; Yamamoto et al. 1993; Carr et al. 2009; Tucker et al. 2011; Li et al. 2012; Sun et al. 2015). Recently, Tucker et al. reported successful rehabilitation of retinal structure and function by transplanting iPSC-derived retinal precursor cells in rhodopsin-null mice (Tucker et al. 2011, 2013). Two different studies demonstrated the potential of native retinal pigment epithelial-transplantation to enhance electroretinogram activity in Royal College of Surgeons (RCS) rats (Lopez et al. 1989; Yamamoto et al. 1993).

In addition, Carr et al. generated functional retinal pigment-epithelial layers from human iPSCs and transplanted them in RCS rats to facilitate short-term maintenance of the photoreceptor-mediated phagocytosis of photoreceptor outer segments (Carr et al. 2009). Sun et al. investigated the therapeutic potential of human iPSC-derived retinal pigment-epithelial cells, neural stem cells, and human mesenchymal stromal cells using *rd1* mice, and the results suggested that iPSC-derived retinal pigment-epithelial cells can slow the deterioration of photoreceptors (Sun et al. 2015). Further, Li et al. showed the integration of administered iPSC-derived retinal pigment-epithelial cells with the resident retinal pigment epithelium of retinal pigment epithelial-65 mouse model resulting in enhanced visual activity (Li et al. 2012).

Given the identical developmental origin of retinal pigment-epithelial and iris pigment-epithelial (IPE) cells, several studies were conducted to administer cultured autologous or freshly isolated IPE cells in rats, rabbits, and nonhuman primates, i.e., monkeys and humans, for the treatment of retinal deteriorating diseases (Sheedlo et al. 1991; Rezai et al. 1997; Abe et al. 1999; Schraermeyer et al. 1999; Thumann et al. 1999; Abe et al. 2000a, b; Lappas et al. 2000; Thumann et al. 2000; Crafoord et al. 2002; Lappas et al. 2004; Aisenbrey et al. 2006). The administration of fresh autologous-IPE cells into the rabbit subretinal region caused no immunological rejection and injected IPE cells exhibited specific-phagocytic activity (Thumann et al. 1999). Importantly, similar results were examined following the administration of autologous cultured IPE-cells in monkeys (Abe et al. 2000a). Moreover, multiple studies employed the autologous IPE-cells to treat subretinal-neovascularization and AMD in humans (Abe et al. 1999, 2000b; Lappas et al. 2000; Thumann et al. 2000). Recently, Thumann et al. administered autologous IPE-cells into the subretinal region of 20 patients exhibiting neovascularization and advanced AMD, which showed improvement of visual acuity without any side effect (Thumann et al. 2000). Likewise, Abe et al. demonstrated similar findings by administering autologous cultured IPE cells into the subretinal region of eight patients with AMD (Abe et al. 1999, 2000b). In contrast, Lappas et al. completed IPE-cell administration in 12 patients with wet-AMD and documented preserved preoperative visual acuity but no improvement after a follow-up period of 6 months (Lappas et al. 2004).

Retinal pigment-epithelial cells derived from hESCs have been employed in multiple human clinical trials (Reardon and Cyranoski 2014; Kimbrel and Lanza 2015; Schwartz et al. 2015, 2016; Song et al. 2015), while only one human clinical study was conducted utilizing iPSC-derived retinal pigment-epithelial cells (Mandai et al. 2017). The iPSC-based autologous cell administration in one patient revealed no harmful effects including immunological rejection and tumor formation, whereas the transplanted retinal pigment epithelial-sheet remained intact with no improvement in best-corrected visual acuity (Mandai et al. 2017). However, retinal pigment-epithelial cells derived from iPSCs were not further administered to other patients since the patient-specific iPSCs harbor multiple genetic lesions that were absent in patient fibroblast cells (Mandai et al. 2017).

Conclusion

In conclusion, we provide the potential of stem cells including nonocular, ocular, and pluripotent stem cells for the treatment of ocular dysfunctions. Ocular diseases such as RP, AMD, diabetic retinopathy, cataractogenesis, uncorrected refractive errors, glaucoma, and corneal dystrophies are responsible for severe visual impairment worldwide. In the near future, the count of individuals with compromised vision is projected to escalate dramatically because of aging and population growth. Stem cells owing to their repair and regeneration ability have immense potential to treat multiple ocular conditions. The eye is an ideal site to examine the therapeutic efficacy of stem cell-based transplantation. The ocular function can be evaluated using standardized outcome measures and in vivo imaging of the retina, lens, and cornea. To this end, multiple stem cell therapies employing cultured and pluripotent stem cell-derived differentiated cells have been investigated as potential treatment options for limbal-stem-cell-deficiency, corneal endothelial dysfunctions, glaucoma, AMD, and other retinal dystrophies in preclinical and clinical settings. A better understanding of the location and function of ocular stem cells has led to the exploration of possible therapeutic options for ocular diseases. However, the evaluation of stem cell treatments in clinical settings regarding long-term complications and technical challenges needs further attention. In conclusion, working on stem cells is one of the sizzling research sectors in biology. Both tissue-resident as well as pluripotent stem cell-derived differentiated cells can prevent vision loss by repairing/replacing the degenerative cells in ocular diseased tissues.

Cross-References

► The Function of Stem Cells in Ocular Homeostasis

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Myoblast Therapies Constitute a Safe and Efficacious Platform Technology of Regenerative Medicine for the Human Health Industry

22

Peter K. Law, Wenbin Li, Qibin Song, Shi Jun Song, Jun Ren, Manye Yao, Qiaoyun Li, Qizhong Shi, Keqiang Wang, Jing Wang, Lei Ye, Jian-Hua Ma, Khawaja Husnain Haider, Li-ping Su, Ping Lu, Weyland Cheng, Ming Zhang Ao, and Danlin M. Law

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P. K. Law (✉)
Cell and Gene Therapies, Cell Therapy Institute, Wuhan, China

Department of Oncology, Renmin Hospital of Wuhan University, Wuhan, China
e-mail: peter@celltherapy.com

W. Li
Capital Medical University, Beijing, China

Q. Song · J. Wang
Renmin Hospital of Wuhan University, Wuhan, China
e-mail: qibinsong@whu.edu.cn

S. J. Song · Q. Shi · K. Wang
Xinxiang Medical University, Henan, China

J. Ren
Tong Ren Hospital of Wuhan University, Wuhan, China

M. Yao · W. Cheng
Children's Hospital Affiliated to Zhengzhou University, Henan Children's Hospital, Zhengzhou Children's Hospital, Zhengzhou, China

Q. Li
Zhengzhou University, Henan, China

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L. Ye · L.-p. Su
Biomedical Engineering, University of Alabama at Birmingham, Birmingham, Alabama, USA

J.-H. Ma
Nanjing Medical University, Nanjing, China

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sru.edu

P. Lu · D. M. Law
Cell Therapy Institute, Wuhan, China

M. Z. Ao
Hua Zhong University of Science and Technology, Wuhan, China
e-mail: aomingzhang@hust.edu.cn

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Abstract

Intramuscular implantation of cultured allogeneic myoblasts derived from pathogen-free muscle biopsies of genetically normal human volunteers demonstrated safety and efficacy in clinical studies of Duchenne muscular dystrophy (DMD), heart failure, ischemic cardiomyopathy, Type-II diabetes, cancer, and aging disfigurement. Through natural cell fusion, donor myoblasts inserted their normal nuclei that supplied the complete human genome to replenish the aberrant gene(s). The replacement gene(s) produced single or multiple gene transcripts, factors, and protein(s) in multiple pathways to effect complementary genetic repair. Donor myoblasts also fused among themselves to form normal myofibers. Applications included diagnostic screening, disease prevention, disease treatment, drug discovery, and selection of superior cell clones for therapies. Only 3-week cyclosporine immunosuppression was necessary to support engraftment, development, and functioning. Improvement in the host included production of repairing structural and regulatory proteins, increases in muscle cell number and function, increases in locomotive capacity, breathing capacity and life span in DMD boys, increases in blood ejection and vascularization in heart failure and ischemic patients, and transfer of biochemicals and ions across the muscle cell membrane in diabetic patients. Intra-tumor implantation of allogeneic human myoblasts induced cancer apoptosis, inhibiting metastasis and tumor growth with cancer patients. FDA currently listed 23 myoblast implantation projects, and EMA listed 6, mostly in Phase II with some in Phase III clinical trials. This unique platform technology, patented for its compositions, methods, and related medical devices of cell/gene

therapies, promised to be of great social and economic values in world health and human services.

Keywords

Anti-aging · Cancer · Cosmetology · DMD · Gene therapy · Heart failure · Human gene therapy · Ischemic cardiomyopathy · Muscular dystrophies · Muscle regeneration · Myoblasts · Somatic cell therapy · Stressed urinary incontinence · Type II diabetes

List of Abbreviations

AF	Ankle plantar flexors
ALT	Alanine transaminase
AMT	Allogeneic myoblast transplantation
ANOVA	One-way analysis of variance
AST	Aspartate aminotransferase
bFGF	Basic fibroblast growth factor
CABG	Coronary artery bypass grafting
CMV	Cytomegalovirus
CsA	Cyclosporine-A
CTRF	Cell Therapy Research Foundation
DMD	Duchenne muscular dystrophy
EDB	Extensor digitorum brevis
Fadu	cancer cells
FI	Fecal incontinence
FM	Fusion medium
GLUT4	Glucose transporter 4
GSV	GLUT4 storage vesicle
HBSAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMGT	Human myoblast genome therapy
IGF-1	Insulin-like growth factor
IND	Investigational New Drug
IRAP	Insulin-regulated aminopeptidase
IRB	Institutional Review Board
KE	Knee extensors
KF	Knee flexors
LBT	Lower body treatment
LC6SP	Large chondroitin-6-sulfate proteoglycan
LD	Lactate dehydrogenase
LVEF	Left ventricular ejection fraction
MDA	Muscular Dystrophy Association Inc.
MHC-1	Major histocompatibility class I

MI	Myocardial infarction
MTT	Myoblast transfer therapy
NIR	Neoplasm inhibition rate
RPR	Syphilis
SCT	Somatic cell therapy
SM	Super medium
SMT	Single muscle treatment
SUI	Stressed urinary incontinence
TMR	Trans-myocardial revascularization
TNF-α	Tumor necrosis factor-α
UBT	Upper body treatment
$VEGF_{165}$	Vascular endothelial growth factor 165
WBT	Whole body treatment

Introduction

Gene Defect: The Original Sin

The Book of Genesis has it that when Eve and Adam ate the fruits of the forbidden tree in Eden, they committed the Original Sin and lost eternal life and happiness. Without tasting the forbidden fruit, their children inherited the Original Sin and needed rebirth to regain what was lost. Gene defects are inherited like the Original Sin. By inheriting these defects, human beings lose even normal life and happiness. Myoblast therapies were pioneered, tested, and established by Professor Peter K. Law's teams and colleagues for most human beings to regain what was or may be lost in health because of gene defects.

Genetic Diseases Account for >80% of Human Death

Genetic diseases are those that have demonstrated gene defects with heritability. In additional to single gene defects causing, for example, Duchenne muscular dystrophies (DMD), many incurable diseases are the result of multiple gene defects and haphazard interactions between them. The latter include top killers of mankind such as cancer, cardiovascular diseases, Type II diabetes, and aging. Together, these fatal and debilitating diseases account for more than 80% of human death, for which the human and health services have provided neither prevention nor effective treatment.

Approaches Towards Treatment

Regenerative medicine using biologics is the medicine of the twenty-first century. Cell therapies regenerate degenerative tissues and enhance organ appearance and functions with little side effects. From the fertilized ovum unto death, the cell is the

basic unit of life in the human body. Whereas numerous molecular genetic pathways have yet to be discovered, advent in cell culture and transplantation techniques has culminated into many somatic cell therapies being practiced. They achieve where drugs, devices, organ transplants, and single gene therapies have failed.

Autografts Cannot Treat Genetic Diseases

The same gene defect(s) reside in every living cell of the genetically defective individual throughout life, since all cells of the body are derived from mitosis of the fertilized eggs. Therefore, hereditary degenerative organs cannot depend on any cell derived from the host body to provide regeneration or rebirth. Allograft is a must for the treatment of hereditary degenerative diseases, and the implanted cells must be derived from genetically normal donors to provide the missing gene(s), and not from abnormal donors. By law, cell therapy forbids transplanting genetically abnormal cells into any human being, including oneself.

Treatment Design and Mechanisms

One unique platform biotechnology distinguishes itself from the rest in terms of demonstrated safety, efficacy, applications, maturity, and intellectual property protection. That is human myoblast genome therapy (HMGT) (Law 2009), also known as myoblast transfer therapy (MTT) (Law 1994). Published reports indicated that the MTT platform technology has applications to treating most if not all the genetic diseases mentioned above, and it is the goal of this chapter to provide the essence.

Why Cell Therapy

The cell is the basic unit of all lives. It is that infinitely small entity which life is made of. In hereditary degenerative diseases such as those mentioned, gene defects cause cells to degenerate and die throughout life. When a DMD boy cannot stand by himself, he has already lost 40% of his skeletal muscle cells in his legs, with the remaining cells in his body continue undergoing hereditary degeneration.

An effective treatment must not only repair degenerating cells but replenish dead cells as well (Law 1994). This has been achieved by the transplantation of genetically normal cells called myoblasts because of their unique ability of natural cell fusion and their unidirectional development to become myofibers. Neither pharmaceutics nor molecular medicine can replenish dead cells. There is very limited evidence that they can repair degenerating cells. Myoblasts are efficient, safe, and universal gene transfer vehicles, being endogenous to the body. Since a foreign gene always exerts its effect on a cell, cell therapy will always be the common pathway to health. After all, cells are what life is made of.

Myoblasts: Nature's Chest of Gene Medicine

Evolution of vertebrate muscular system over the past 500 million years witnesses some unique characteristics developed. In human beings, myoblasts are the only cell type which divide extensively without tumorigenicity, migrate, fuse naturally to form syncytia, lose their major histocompatibility class I (MHC-1) antigens soon after fusion, and develop to occupy 55% of the body weight. These combined properties render myoblasts ideal for gene transfer and somatic cell therapy (SCT).

Myoblasts is a living biologic native to the human body, a bioactive product of 500 million years of vertebrate evolution. Each of their nuclei contains the software and hardware of the complete human genome, readily to be switched on and off towards normal muscle development. Because of the vast biodistribution of striated, cardiac, and smooth muscles, any gene defect affecting the myo-genome will have serious consequences, such as found in aging, muscular dystrophy, Type II diabetes, and their related cardiomyopathies.

From human standpoint with 3000 years of recorded medical knowledge, understanding the etiology and pathogenesis will have great bearing in devising preventions and treatments for various muscle diseases. From other mammals' standpoint without any medical capability, myoblasts and other biologics are all they have. The skeletal muscle, being externally located in the body, has developed specific regenerative capabilities and characteristics for frontline defense against predation and diseases, traversing much longer time than human knowledge of medicine.

All the unique properties or specializations, especially the natural cell fusion of myoblasts during muscle development and regeneration, were fully exploited by Law in his pioneer engineering of the MTT platform technology. It should be mentioned that satellite cells, muscle stem cells, and muscle progenitors are essentially myoblasts and have very similar if not exactly the same cell properties.

MTT Platform Technology

MTT is a combined SCT and gene therapy: replenish dead myofibers with live ones, and genetically repairing degenerative myofibers. Intramuscular implantation of cultured allogeneic myoblasts derived from pathogen-free muscle biopsies of genetically normal male volunteers demonstrated safety and efficacy in clinical studies of DMD, heart failure, ischemic cardiomyopathy, Type-II diabetes, cancer, and aging disfigurement.

The MTT invention relates to compositions and methods for preventing and treating mammalian disease conditions that are debilitating, fatal, hereditary, degenerative, and/or undesirable. More specifically, the MTT invention relates to the transplantation of normal, or genetically transduced, or cytokine-converted myogenic cells into malfunctioning, and/or degenerative tissues or organs.

Replenishing Dead Cells

With the immense wisdom and knowledge of human race, scientists have not been able to produce a living cell from nonliving ingredients such as DNA, RNA, ions, carbohydrate, fat, protein, and/or biomaterials. The testing and establishment of MTT concept involved development of two methods between 1975 and 1990: Cell Culture is the only method known to man for the replication of cells in vitro. With proper techniques and precautions, normal myoblasts can be cultured in significant quality and quantity to repair, and to replenish degenerated and damaged myofibers.

Cell transplantation bridges the gap between in vitro and in vivo systems, and allows propagation of "new life" in degenerative tissues or organs of the genetically defective or injured body. Cell transplantation techniques have been developed to ensure donor myoblast survival, development, and functioning. After transplantation, donor myoblasts fused among themselves to form multinucleated myotubes that developed into normal myofibers to replenish degenerated myofibers and lost function (Law 1994).

Repairing Hereditary Degenerative Cells

Cell fusion transfers the nucleus, thus all the normal genes within, like delivering a gene medicine chest to the abnormal myofiber. It is important to recognize that, for proper gene regulation and expression, the DNA software packaged in the chromosomes needs other cell organelles as hardware to operate, with spatial and temporal controls.

Correction of a gene defect occurs spontaneously at the cellular level after cell fusion. The natural integration, regulation, and expression of the full complement of over 30,000 normal genes impart the normal phenotypes onto the heterokaryon. Protein(s) or factor(s) that were not produced by the host genome because of the genetic defect are now produced by the normal genome. Cytokines and cofactors derived from expression of other genes at different time and space all corroborate to restore spontaneously the normal phenotype of the degenerating cell. The latter information is often unknown to the molecular geneticists, thus accounting for the limited success of molecular medicine. In MTT, genome of the donor myoblast operates naturally as usual, but in a multinucleated, heterokaryotic myotube mosaic with the normal and the abnormal nuclei. It is in such manipulated, genetically engineered environment that phenotype correction of gene defect occurs.

Engineering Genetic Mosaicism with MTT: Supply of Normal Genome via Injection of Myoblasts

Myofibers of DMD patients contain genetically dystrophic nuclei each of which lacks the normal dystrophin gene. In MTT, the injection trauma activates the muscle regenerative processes. Through natural cell fusion, donor myoblasts convey their normal nuclei that supply the complete human genome, carrying normal copies of the dystrophin gene into the regenerative myofibers or myotubes. The foreign

dystrophin gene corroborate with various cofactors to produce gene transcripts and the missing protein dystrophin, resulting in complementary genetic repair of the DMD myofiber phenotype (Law et al. 1990a, 1998).

"Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to prevent or to treat diseases" (Kessler et al. 1993). This FDA definition placed MTT into the field of gene therapy. Genetic mosaicism describes the mixture of genetically normal and dystrophic nuclei coexisting in a myofiber or a myotube in the MTT treated host muscle. This type of myofibers is termed mosaic myofiber, being mosaic with normal and dystrophic nuclei. There is no gene editing. There is no hybridization at the chromosomal level. Every cell is a diploid and not tetraploid.

Genetic mosaicism also describes the mixture of genetically normal, dystrophic, and mosaic myofibers coexisting in the host muscle after MTT. The normal myofibers are newly formed from natural fusion of the donor myoblasts with each other.

Original Testing of Idea

Although the role of myoblasts/satellite cells in myogenesis and muscle regeneration dated back to the early 1960s (Konigsberg 1963; Mauro 1961), the use in animal therapy was not reported until 1978 (Law 1978). The original idea of MTT dated back to 1975, and experiments testing MTT were first published by Law in 1978 (Law 1978). A deliberate attempt was made in adult dystrophic mice to produce mosaic muscles containing normal, dystrophic, and mosaic myofibers from the regenerates of normal and dystrophic minced muscle mixes. The mince and mix procedures were designed to provide an environment for the intermingling and fusion of normal and dystrophic satellite cells. These muscle regenerates produced greater twitch and tetanus tensions and more normal appearing cell structure than control regenerates of minced dystrophic muscles. It was concluded that the introduction of cell contents of normal genotype into dystrophic muscles could improve the function of the latter (Law 1978). Additional proof-of-concept animal studies are described in the section "Pathogenesis: Cell Membrane Defect."

Pertinent Muscle Developmental Biology

Mammalian skeletal muscles are derived from the mesodermal germ layer in the embryo. In human, mesoderm first appears at 20 days after fertilization. Concomitant is the appearance of somite that increase in number with time. Within the somite are uncommitted mitotic precursor cells capable of giving rise to muscle, bone, cartilage, blood, lymphatics, and connective tissues. The commitment to being myogenic occurs early on since myoblasts, as these cells are called, are found in the limb buds at 26 days of gestation. Satellite cells which are myoblast reserves in adult

muscles contain actin-like filaments in their cytoplasm. Such lineage determination is influenced by embryonic induction and irreversible gene expression (Jacobson 1970).

Myogenesis

The developmental processes of myoblasts, myotubes, myofibers, and satellite cells, especially the genetically programming of cell fusion are products of 500 million years of vertebrate evolution. Lack of human fetal tissues for research had deemed myogenesis be studied in vitro. Beginning as small spheres of about 10–12 μm in diameter, myoblasts grow best on collagen attachment which resembles the extracellular matrix in vivo. Under proper cell culture condition, the transformation into spindle-shaped cells occurs within 3 days.

Myoblasts are characterized by their abilities to divide, migrate, align, and fuse to form multinucleated myotubes. Cell division is an intrinsic property of myoblasts as evident by myoblast proliferation in serum-free culture media. However, proliferation over one billion myoblasts from primary culture of 2 g of muscle biopsy requires growth factors. Myoblasts will continue to proliferate for as long as nutrients, adherent space, temperature, and pH are optimal. It is known that myoblasts do not fuse when they are in the S, G2, M, or even the early G1 phases of the cell cycle. Although it is not possible to study division synchronization in the human fetus, the myoblast doubling time in culture is 24–40 h, depending on the regenerative vigor of the satellite cells of the donor. Myoblasts reportedly could undergo 80 mitotic divisions. In our laboratory, myoblasts lost their ability to fuse after 50 generations of proliferation.

A built-in regenerative measure resides with the satellite cells that are basically myoblast reserves found in adult skeletal muscle fibers. These are mononucleated cells located between the basal lamina and the plasma membrane of myofibers. Satellite cells exhibit the same characteristics as myoblasts. Myotubes are formed from natural fusion of myoblasts. Each of them contains 200–500 nuclei, a uniquely large genomic store and manufacture plant for producing and depositing contractile filaments such as actin, myosin, myoglobin, and tropomyosin, and related molecules in an organized manner to form sarcomeres of myofibers. Myotubes can easily be identified by immunologically stained positively for heavy meromyosin. Equipped with these structural and biochemical bases of contraction, some myotubes can contract spontaneously in culture, and eventually pull from the collagen surface. Without neural induction or nerve innervation, the advance myotubes will undergo degeneration and die within 10 days. The myotubes will not develop into a myofiber unless innervated by a motor axon.

Myofibers are formed from developing myotubes when the contractile proteins are structured into filaments and packaged into basic contracting units called the sarcomeres. Hundreds of sarcomeres are aligned to form a myofibril many of which comprise a myofiber. Each sarcomere is made up of myosin and actin filaments linked together by cross-bridges.

Neuromuscular Transmission

With the arrival of the axonal terminal, an area of the myotube membrane which is adjacent to the axonal terminal becomes thickened and convoluted due to the formation of large amounts of acetylcholine receptors and ionic channels. The mature neuromuscular junction is a highly specialized relay station, transmitting the central command to generate contraction of the myofiber. Neuromuscular transmission is through the release of the neuromuscular transmitter acetylcholine which, upon combining with their postjunctional receptors, is metabolized by cholinesterase and produces significant membrane depolarization to generate excitation-contraction. These junctions stain positively for acetylcholinesterase.

Excitation Contraction Coupling

Postjunctional membrane depolarization of more than 40 mv will trigger an action potential to propagate the transverse tubular system, releasing Ca^{2+} from the sarcoplasmic reticulum, triggering millions of cross-bridges to be formed, sliding the actin filaments toward the center of each myosin filament, and results in force generation. Contraction and relaxation require Ca^{2+} and ATP.

Number of Myofibers and Strength

As the bones elongate during puberty, the passive stretch induces additional sarcomere production with subsequent increase in strength. Although the number of myofibers remains constant for individual muscles, the number of myofibrils within the myofiber can vary according to genetic and environmental differences. As more contractile proteins are deposited and the sarcoplasmic reticulum better developed, the centrally located nuclei migrate peripherally.

Numerous structural proteins and glycoproteins are synthesized and deposited during the transition from myotube to myofiber. Among these is dystrophin, a surface membrane protein which is not present in the myofibers of DMD (Hoffman et al. 1987) as a result of the gene defect (Monaco et al. 1986; Koenig et al. 1987).

Parameters Governing Cell Fusion

A condition called serum deprivation or a sudden reduction in serum concentration will precipitate cell fusion in culture. Cell fusion occurs only after the myoblasts have undergone considerable cycles of division and are withdrawn from the mitotic cycle (Banker et al. 1971). In the human fetus, myoblasts within a somite are

essentially replicating clones. Close confinement within the somite ensures that the myoblasts are always at a state of confluence. The latter is a prerequisite for cell fusion. The decision to fuse in vivo is genetically programmed, and fusion occurs without any neural contact or influence. It involves structural gene transcription and RNA synthesis, especially in the formation of the receptors on the myoblast surface that are responsible for cell recognition, cell adhesion, and membrane restructuring (Pearson and Epstein 1982; Davidson 1976). The end products are multinucleated myotubes each of which is formed from fusion of 200 or more myoblasts.

Controlled Cell Fusion

Through unknown mechanisms, the MHC class 1 surface antigens of myoblasts are very mildly expressed after cell fusion and the myotubes so formed exhibit minimal, if any, antigenicity. To foster donor cell survival and development, it would be useful to be able to control, initiate, or facilitate cell fusion once myoblasts are injected. This strategy was engineered to reduce the time of immunosuppression necessary for engraftment of allogeneic myoblasts.

As the myoblasts are injected intramuscularly, injection trauma causes the release of basic fibroblast growth factor (bFGF) and large chondroitin-6-sulfate proteoglycan (LC6SP). These latter growth factors stimulate myoblast proliferation. Unfortunately, they also stimulate the proliferation of fibroblasts that are already present in increased amount in the dystrophic muscle. That is why it is necessary to inject as pure as possible fractions of myoblasts in MTT without contaminating fibroblasts.

Controlled cell fusion can be achieved by artificially increasing the concentration of LC6SP over the endogenous level. LC6SP stimulates cell proliferation towards confluency, thus facilitates cell fusion. In addition, insulin or insulin-like growth factor I (IGF-1) facilitates the developmental process, resulting in the formation of myotubes soon after myoblast injection. Although the use of bFGF, LC6SP, and IGF-1 at optimal concentrations in the injection medium may lead to greater MTT success, inclusion of one or more of these factors would introduce immunologic complications.

Myoblasts fuse readily at low serum concentration in culture. At the end, it was an inventive step introduced exploiting serum deprivation that was found most effective. This involved exposing the myoblasts to 100% host serum as the last step in cell production, a step that provided enough nutrients to sustain cell survival and vigor before implantation. The carrier solution could consist of 100% host serum, or it could be supplemented with human albumin and injection saline when enough host serum was not available. Intramuscular injection of myoblasts dramatically reduced the serum concentration and exposed the myoblasts to intercellular fluid within the perimysium, a condition constituting serum deprivation. This controlled cell fusion procedure supported myoblast survival and development into myotubes in 7–10 days after MTT.

Mitotic Cardiomyocytes via In Vitro Controlled Cell Fusion

Whereas MTT results in the formation of genetic mosaicism with gene transfer occurring in vivo, the production of heterokaryons in vitro has immense medical application. This can be achieved by controlled cell fusion with myoblasts.

The original program of research relates to the in culture transfer of the normal nuclei carrying the regenerative genome from donor myoblasts into the genetically normal and/or abnormal cardiomyocytes. This development is especially important considering that cardiomyopathic symptoms develop in mid adolescence in about 10% of the DMD population. By age 18, all DMD individuals develop cardiomyopathy. Undoubtedly, the ability to replenish degenerated and degenerating cardiomyocytes will have immense impact on heart diseases even in the normal population where there is a great shortage of hearts for transplantation.

Normal cardiomyocytes exhibit very limited ability to proliferate in vivo or in vitro. The heart muscles damaged in heart attacks or in hereditary cardiomyopathy cannot repair themselves through regeneration. It is envisioned that the integration of mitosis, an extremely efficient characteristic of myoblasts, will enable the heterokaryotic cardiomyocytes to proliferate in vitro. Controlled cell fusion between normal myoblasts and normal cardiomyocytes may result in heterokaryons exhibiting the characteristics of both parental myogenic cell types. Clones can be selected based on their abilities to undergo mitosis in vitro, to develop desmosomes, gap junctions, and to contract strongly in synchrony after cell transplantation. These genetically superior cells can then be delivered through catheter pathways after mapping of the injured sites. With the ability to grow large quantity of these cardiomyocytes, the correction of structural, electrical, and contractile abnormalities in cardiomyopathy can be tested first in cardiomyopathic hamsters, ischemic porcine model, and if safe and effective, in humans.

Injection Methods Regulate Cell Distribution and Fusion

The biodistribution of donor myoblasts and the integration of donor nuclei through cell fusion are of supreme importance towards MTT success. Aside from donor cell survival in an immunologically hostile host, cell fusion is the key to strengthening dystrophic muscles with MTT. To improve the fusion rate between host and donor cells, various injection methods aimed at wide dissemination of donor myoblasts were tested and compared. The goal was to achieve maximum cell fusion with the least number of injections.

Methods of myoblast delivery included injecting perpendicular to the muscle fiber surface, parallel to myofibers, and diagonally traversing the myofibers. Myoblasts were infused slowly as the needle was withdrawn. Results indicated that myoblasts injected oblique to myofiber orientation were widely and evenly distributed. Myoblasts injected perpendicular to myofiber orientation were partially distributed. Myoblasts injected longitudinally through the core of the muscles and parallel to the myofibers were poorly distributed (Law et al. 1994a).

Cyclosporine Immunosuppression

When human myoblasts were transplanted into ischemic porcine ventricle, there was a transient elevation of porcine antihuman-myoblast antibodies at 1 week after the xenograft (Haider et al. 2004a, b). The antibody level subsided at the second week after MTT, indicating that no more than 2 weeks of cyclosporine immunosuppression would be necessary for human/pig xenografts or for human allografts. Because of the low antigenicity of allogeneic myoblasts, the only immunosuppressive agent used was cyclosporine, at a dose of 5–7 mg/kg body weight divided into two daily doses. The dosage was varied to maintain serum trough concentrations in the range 100–150 ng/ml. With the newly developed controlled cell fusion procedure, cyclosporine immunosuppression has been reduced from 3 months to 3 weeks, beginning at 3 days before MTT and weaning at half dosage at the beginning of the third week.

Muscular Dystrophy

Muscular dystrophy is a group of inherited diseases characterized by progressive degeneration of skeletal muscles. The word "dystrophy," implicating malnutrition, has been in use since the eighteenth century. At the dawn of Neurosciences in the early 1970s, McComas postulated the neural hypothesis of muscular dystrophy (McComas et al. 1971). Its basic concept was that motoneurons did not supply normal "trophic" substance(s) to maintain muscle fibers in a healthy state.

Etiology

Law and Atwood (1972a) had previously reported, within single mice, the slow contracting soleus muscle being cross-reinnervated by the nerve of the fast contracting flexor digitorum longus. Whereas physiological conversion of the slow muscle by the "fast" nerve was noted in all normal preparations, a similar conversion was noted in the soleus of only one of 10 dystrophic animals. The results were interpreted to mean that either some "neurotrophic" factors were absent in the nerves of dystrophic mice or that the dystrophic muscle was not capable of responding to existing "neurotrophic" factors.

In a continuation study, Law et al. reported parabiosis being established between pairs of dystrophic mice and normal littermates with a cross of the "fast" tibial nerve of one partner onto the slow soleus muscle of the other. This was a "double cross" preparation, such that each parabiont had a soleus muscle cross reinnervated by a tibial nerve of its partner. This unique preparation allowed the investigators (a) to monitor the "neurotrophic" influence of a "fast" nerve on a slow muscle, (b) to assess specifically the influence of the nerve on the expression of dystrophy (Law et al. 1976a), and (c) "myotrophic" influence on motoneurons (Law 1977).

Electrophysiological, cytochemical, and structural analyses indicated that the crossed "fast" nerves of normal or of dystrophic genotype were effective in altering

the cytochemical pattern of the slow muscles to fiber types' characteristic of fast muscles. However, normal nerves innervating solei of the dystrophic parabiont did not arrest the progress of the disease, and nerves of dystrophic genotype innervating muscles of the normal parabiont did not induce a pathological state. The results indicated that the peripheral motor nerves of dystrophic mice were normal in exerting "neurotrophic" influences and that muscular dystrophy progresses despite the presence of normal "neurotrophic" influences. This unique approach of a double nerve cross achieved through parabiosis provided strong evidence that the etiology of hereditary muscular dystrophy in this species was not nerve mediated. The results nullified the hypothesis that a "neurotrophic" deficiency was the primary manifestation of the defective gene in hereditary muscular dystrophy of mice. The findings that the dystrophic nervous system was normal in its ability to form synapses, to induce fiber type differentiation, to bring myofibers to maturity, and to maintain the structure and function of a normal muscle are significant, not only in terms of understanding of the etiology of muscular dystrophy, but also of developing transplant treatments (Law et al. 1976a). Without such knowledge, it would be imprudent to attempt strengthening dystrophic muscles with normal myogenic cell transfer.

Furthermore, a detrimental "myotrophic" influence was found to be exerted on motoneurons in dystrophic mice. Dystrophic solei reinnervated by nerves of normal genotype showed a 23% reduction in number (17 ± 2, mean ± SD) and a 50% reduction in size (78 ± 17 mg, mean ± SD) of motor units as compared to the normal solei reinnervated by nerves of dystrophic genotype (22 ± 1, 154 ± 38 mg, mean ± SD). The former preparations also showed abnormal endplates compared to the latter. These endplates were irregular in size and shape and often exhibited decreased acetylcholinesterase activity (Law 1977; Saito et al. 1983).

Pathogenesis: Cell Membrane Defect

The first direct evidence of membrane abnormality in mammalian dystrophy was reported in 1972 (Law and Atwood 1972b). Subthreshold direct stimulation elicited a smaller response from the dystrophic fiber than from the normal one, indicating that the dystrophic fiber was "leaky" to ionic currents. In a later study, about 55% of dystrophic myofibers examined were abnormal in their ability to generate action potentials. Some of these fibers showed no detectable response to supramaximal stimulation of the motor nerve, whereas in others, abortive spikes or localized end-plate potentials were recorded. The proportion of fibers showing very small or no electrical response increased when recordings were made away from the end-plate zone. The abortive spikes indicated a structural defect in the sarcolemma such that sodium conductance was diminished (Law et al. 1976b).

The abortive spikes did not propagate the whole length of the myofibers such that sarcomeres further away from the motor endplates would not receive depolarization to contract and would be "wasted." Dystrophic myofibers with abortive spikes generated localized contraction close to the endplates but the tensions developed were lower than normal. When given a normal resting potential, hyperpolarization to

−80 mV of fibers with abortive spikes could not cause the indirectly elicited abortive spikes to increase in amplitude. This indicated that the abortive spikes were the result of membrane "leakiness" rather than reduced membrane hyperpolarization. Furthermore, when these fibers were subjected to the passive stretch of the antagonistic muscle and the active pull of the localized contraction, breach in the plasma membrane (Law et al. 1983) occurred at the region of nonactivated sarcomeres.

With leakage of Ca^{2+} into the myofibers, there are mitochondrial Ca^{2+} overload and localized hypercontraction. Also, Ca^{2+}-activated neutral proteases and lysosomal activities are activated resulting in muscle necrosis. Some fibers showed positive acid phosphatase reaction in muscle nuclei, mitochondria, sarcoplasmic reticulum, and contractile myofilaments. Membrane defect was found not only in the sarcolemma but also in the sarcoplasmic reticulum and the nucleus. Eventually, the contractile elements are replaced with connective tissue (Law et al. 1983, 1990b).

To conclude, the dystrophic gene is transcribed into a structural protein abnormality which directly or indirectly results in membrane "leakiness." The establishment of an abnormal ionic equilibrium across the membrane especially that of Na^{+}, constitutes the earliest detectable pathophysiology in mediating weakness and necrosis of dystrophic myofibers (Law 1980). It was not until 1987 that dystrophin was identified as the structural protein defect in the sarcolemma of DMD myofibers.

Duchenne Muscular Dystrophy

Natural History

DMD manifests as a continuous muscle degeneration and loss of strength beginning at 1.5 years of age and lasting throughout life (Ziter et al. 1977; Brooke et al. 1983; Mendell et al. 1989; Fenichel et al. 1991). Growth outstrips the disease progress between ages 3 and 5, giving a false impression of remission; otherwise deterioration is continuous (Roelofs et al. 1979; Walton and Gardner-Medwin 1981). Degeneration is more severe in the proximal and anti-gravitational muscles than distal ones (Roelofs et al. 1979; Walton and Gardner-Medwin 1981) and proximal muscle weakness in the lower body is responsible for the Gowers' sign used in physical diagnosis.

Debilitating and fatal, DMD affects 1 in 3300 live male births (Emery 1991). DMD boys usually lose 50% of the strength in their leg muscles by age 9, and are wheelchair-bound by age 12. Three-quarters die before age 20. Pneumonia usually is the immediate cause of death, with underlying respiratory muscle degeneration, failure to inhale enough oxygen and to expel lung infection. Cardiomyopathic symptoms develop in mid-adolescence (Engel 1986) in about 10% of the DMD population. By age 18, all DMD individuals develop cardiomyopathy (Nigro et al. 1990), but cardiac failure is seldom the primary cause of death (Walton and Gardner-Medwin 1981).

Strategizing DMD Treatment

Human race is far from curing any genetic disease, not only from the practical standpoint but also conceptually how to convert the abnormal genome back to normal again. Perhaps the best alternative to sustain normal function was to engineer genetic mosaicism into muscles of the DMD boy as found in the X-linked carrier, the boy's mother. According to the Lyon hypothesis (Lyon 1961), random inactivation of one or the other X-chromosome can occur in any nucleus throughout the body of a Duchenne female carrier; individual myofibers would therefore contain mixtures of normal and dystrophic nuclei similar to the mosaic fibers produced by injecting normal myoblasts into dystrophic muscles (Law et al. 1990c). The mosaic myofibers exhibit normal phenotype, presumably due to metabolic or developmental complementation as a result of sharing the normal nuclei and therefore the normal genes. In addition, muscles of chimeric mice containing fibers of normal, dystrophic, and mosaic genotypes exhibit normal function (Law and Yap 1980; Peterson 1974).

It thus appeared that the logical and practical approach to reverse the expression of dystrophy in DMD muscles was to induce genetic mosaicism with the incorporation of the normal nuclei through MTT. Natural transduction with the normal nuclei ensured orderly replacement of dystrophin and related proteins at the cellular level in DMD. This ideal gene transfer procedure is unique to muscle. After all, only myoblasts could fuse and only myofibers were multinucleated in the human body. By harnessing these intrinsic properties, MTT transferred all normal genes to effect genetic repair, without the need to identify which gene was abnormal and which protein was missing (Law et al. 1990a).

Induction of Genetic Mosaicism

Normal and Dystrophic Minced Muscle Mixes

The foremost study in adult dystrophic mice was aimed at producing mosaic muscles containing normal, dystrophic, and mosaic myofibers from the normal and dystrophic minced muscle mixes (Law 1978). It focused on incorporating the "missing" gene and its product(s) into genetically defective cells through cell transplantation and natural cell fusion, the result of which is strengthened dystrophic muscles.

Newborn Muscle Transplant

In a later study, muscles of newborn normal mice were grafted into recipient soleus muscles of dystrophic mice. Results obtained 6 months after the grafting indicated that the grafts survived, developed, and functioned in the dystrophic environment. The regenerates had larger cross-sectional areas and more muscle fibers than the contralateral dystrophic solei. MTT increased the mean twitch tension of adult dystrophic muscles to that of the normal (Law and Yap 1979). The concept of replenishing lost cells and repairing degenerative cells through the production of genetic mosaicism using MTT was firmly established (Law and Yap 1979).

Mesenchymal Cell Transplant

MTT with myoblasts became the logical development since myoblasts do not require neuronal and capillary connections to survive and develop, and since myoblasts can fuse to effect genetic repair. A convenient way to obtain normal myoblasts in mice is through dissection of limb-bud mesenchyme of day-12 embryos. Dissected mesenchyme was surgically implanted into the solei of dy2Jdy2J mice. Host and donors were histocompatible. Contralateral solei served as controls. Six to seven months postoperatively, the myoblast-implanted solei exhibited greater cross-sectional area, total fiber number, better cell structure, and twitch and tetanus tensions than their contralateral controls (Law 1982).

MTT with Primary Myoblast Culture

In 1988, primary myoblast cultures from limb-bud explants of normal mouse embryos were injected into the soleus muscles of histocompatible dystrophic hosts (Law et al. 1988a). In addition, clones of normal myoblasts were injected into the leg and intercostal muscles of histoincompatible hosts, using cyclosporine-A (CsA) as a host immunosuppressant (Law et al. 1988b). Using glucose phosphate isomerases (GPI) as genotype markers, donor myoblasts were shown to have fused among themselves and developed into normal myofibers. They also fused with dystrophic host myogenic cells to form mosaic myofibers of normal phenotype (Law et al. 1988a, b, 1990b, c). These two mechanisms of genetic complementation were shown to be responsible for improvement in muscle genetics, structure, function, and animal behavior of the test dystrophic mice. Prolongation of the life spans of the myoblast-injected dystrophic mice was demonstrated (Law et al. 1988a, b, 1990b, c). The improvement persisted despite cyclosporine-A withdrawal.

With the discovery of the mdx mouse dystrophy model, and of the absence of the gene product dystrophin being the membrane defect of DMD (Hoffman et al. 1987), a new biochemical marker became available to demonstrate MTT efficacy (Partridge et al. 1989; Karpati et al. 1989; Chen et al. 1992). With implantation of cultured normal myoblasts into muscles of immunosuppressed mdx mice, MTT was shown to convert mdx myofibers from dystrophin-negative to dystrophin-positive (Partridge et al. 1989; Karpati et al. 1989; Chen et al. 1992). The study demonstrated biochemical improvements in the mdx mouse model, an additional evidence to confirm the efficacy of MTT.

MTT in DMD Subjects

All critical animal experimentation had been completed (Law et al. 1990b; Law 1990) when we began the MTT clinical trial. Phase I MTT began on February 15, 1990 (Law et al. 1990a) after approval from four institutional review boards. It was the first human gene therapy and the first somatic cell therapy clinical trial as reported by Hooper (1990). Cultured human myoblasts were used as vehicles to deliver the normal genome into DMD myofibers to repair genetic defects. As a cell therapy,

MTT was to replenish the degenerated myofibers also. The clinical trials were based on the safety and efficacy of MTT previously demonstrated in the dydy, $dy^{2J}dy^{2J}$, and mdx mice (Law 1978, 1982; Law et al. 1988a, b, 1990b, c; Law and Yap 1979; Partridge et al. 1989; Karpati et al. 1989; Chen et al. 1992).

A pioneering work (Anderson 1990, 1992, 1995; Beardsley 1990) has often been considered as the "first human gene therapy." Correction of the ADA deficiency study began on September 14, 1990 (Anderson 1990, 1992), 2 months after the MTT correction of the DMD gene defect was published (Law et al. 1990a).

Single Muscle Treatment (SMT): Phase I Trial

The randomized, double-blind study aimed to determine the survival, development, and functioning of donor myoblasts in dystrophic muscles of 11 DMD boys. We hypothesized that intramuscular injection of normal myoblasts could significantly improve the biochemistry, structure, and function of dystrophic muscles. Subjects and parents gave informed consents.

The safety and efficacy of MTT was assessed by injecting the left extensor digitorum brevis (EDB) muscle of a 9-year-old DMD boy with about 8×10^6 myoblasts. Donor myoblasts were cloned from satellite cells derived from a 1 g rectus femoris biopsy of the normal, adoptive father. Cyclosporine was administered for 3 months at a dose of 5–7 mg/kg body weight divided into two daily oral doses. Donor myoblasts survived, developed, and produced dystrophin in myofibers biopsied from the myoblast-injected EDB 92 days later. Dystrophin was not found in the contralateral sham-injected muscle. This first case, published in Lancet on July 14, 1990, suggested that MTT offered a safe and effective means for alleviating biochemical deficit(s) inherent in muscles of DMD (Law et al. 1990a).

Six years after the foremost MTT, dystrophin was found in the myoblast-injected muscle but not in the sham-injected muscle (Law et al. 1997a). Six years was the longest period through which any gene therapy had sustained positive results. Despite cyclosporine withdrawal at 3 months after MTT, myofibers expressing foreign dystrophin were not rejected. This was because dystrophin was present in the inner surface of the plasma membrane, and because mature myofibers did not exhibit MHC class 1 surface antigens. Not only had the result demonstrated MTT overall safety and efficacy in this single case, it also showed stability in the integration, regulation, and expression of the inserted dystrophin gene. The presence of dystrophin in the myoblast-injected but not in the sham-injected muscle provided unequivocal evidence of the survival and development of donor myoblasts in the myoblast-injected muscle.

SMT Safety

At no time during the 92 days after myoblast injection of the 11 subjects were there any sign of erythema, swelling, or tenderness at the injection sites. Serial laboratory evaluation, including electrolytes, creatinine, and urea, did not reveal any significant changes before or after MTT. There was no clinical evidence of an adverse reaction to MTT or to Cy (Law et al. 1990a).

SMT Efficacy

Myoblast-injected EDBs showed increases in maximal contractile force whereas sham-injected EDBs showed reductions (Law et al. 1991a, b, c). Both immunocytochemical staining and immunoblot revealed dystrophin in the myoblast-injected EDBs. Dystrophic characteristics such as fiber splitting, central nucleation, phagocytic necrosis, variation in fiber shape and size, and infiltration of fat and connective tissues were less frequently observed in these muscles (Law et al. 1991a, b, c).

SMT Significance

The first MTT on a DMD boy on Feb. 15, 1990 (Law et al. 1990a) marked the first clinical trial on human gene therapy (Hooper 1990). Its success was reported (Law et al. 1990a; Kolata 1990). MTT inserted, through natural cell fusion, all the normal genes within the nuclei of the donor myoblasts into the dystrophic myofibers to repair them. In addition, donor myoblasts also fused among themselves, forming genetically normal myofibers to replenish degenerated ones. Thus, full complements of normal genes were integrated, through a natural developmental process of regeneration, into the abnormal cells and into the abnormal organ to effect genetic cell therapy.

The US Patent Office issued to Law a pioneering patent (U.S. Pat. No. 5,130,141) entitled "Composition for and methods of treating muscle degeneration and weakness" on July 14, 1992.

In October 1993, the FDA officially began regulating SCT with a definition of "autologous, allogenic, or xenogeneic cells that have been propagated, expanded, selected, pharmacologically treated, or otherwise altered in biological characteristics ex vivo to be administered to humans and applicable to the prevention, treatment, cure, diagnosis, or mitigation of disease or injuries" (Federal Registrar 1993). MTT fell under the umbrella of SCT and myoblasts and its physical, genetic, or chemical derivatives became potential biologics in the treatment of mammalian diseases.

On May 25, 1994, FDA granted permission for Cell Therapy Research Foundation (CTRF) to charge $63,806 per subject. CTRF was a nonprofit 501 (c) (3) research foundation founded in 1991 by Peter K. Law, the MTT inventor. Authorization by the FDA for CTRF to recover costs from subjects of these clinical trials was extremely important to establish the scientific credibility MTT and CTRF deserved, quoting FDA spokesman Monica Revelle, "Permission to bill for an Investigational product is granted rarely," "Applicants must endure numerous procedures, and must have what looks like a viable product at the end of the rainbow. It's used mainly to support testing of promising technology by small companies." (Shepard 1994).

At the time CTRF held the first and only FDAapproved human clinical trial under an Investigational New Drug (IND) application on MTT. It is extremely important to realize that CTRF had been working closely with the FDA to establish criteria and policies in the approval process of this IND for genetic cell therapy. The use of viral vector mediated gene therapy on human neuromuscular diseases had not met FDA approval.

Lower Body Treatment (LBT): Phase II Trial

This was the first attempt to improve the locomotive ability and thus the quality of life of DMD patients using MTT. The LBT protocol received approval from the Essex Institutional Review Board Inc. in Lebanon, NJ, and from the Patient Participation Committee of the Baptist Memorial Hospital Medical Center in Memphis, TN. Both were in compliant with the regulations of the FDA. The SMT and the LBT studies had long begun before the FDA established policies and regulations for cell/gene therapies in October 1993. Whereas the patient families were in great support of CTRF and MTT according to Dr. Thomas E. Furlong's letter in Science (Law 1992), the Muscular Dystrophy Association Inc. (MDA) was attempting to derail CTRF's MTT program through its association with the Appropriation Committee Chairman of the US Congress acting on NIH and FDA (Law 1992, 1993).

The feasibility, safety, and efficacy MTT were assessed in an experimental lower body treatment (LBT) involving 32 DMD boys aged 6–14 years, half of whom were nonambulatory. Through 48 injections, five billion (at concentration of 55.6×10^6/mL) normal myoblasts were transferred into 22 major muscles in both lower limbs, in 10 min with the subject under general anesthesia (Law et al. 1992). Ten subjects received myoblasts cultured from satellite cells derived from 1-g fresh muscle biopsies of normal males aged 9–21 years. Donor myoblasts for the remaining 22 boys were subcultured from reserves frozen 1 month to 1.5 years ago. Only four donors were known to have identical histocompatibility with their recipients. All subjects took oral doses of the immunosuppressant cyclosporine, beginning at 2 days before MTT and lasting for 6 months after MTT to facilitate donor cell survival.

There was no evidence of an adverse reaction to MTT or cyclosporine as determined by serial laboratory evaluations including electrolytes, creatinine, and urea. This was the first study, using conservative and objective quantitative analyses, to have produced a statistically significant 50% increase in strength in 36% of 180 muscle groups in 31 DMD boys, despite a 9-month period of dystrophy progression. In addition, 25% of the muscle groups were as strong as they were 9 months ago.

Objective functional tests using the KinCom Robotic Dynamometer measured the maximum isometric contractile forces of the ankle plantar flexors (AF), knee flexors (KF), and knee extensors (KE) before MTT and at 3, 6, and 9 months after MTT. The AF, being distal muscles and less degenerative than the KE and the KF, showed no decrease in mean contractile force 3 months after MTT, and progressive increases in force at 6 and 9 months after MTT. At 9 months after MTT, 60% of the 60 AF examined showed a mean increase of 50% in force; 28% showed no change; and only 12% showed a mean decrease in force of 29% when compared to the function of the same muscles before MTT. The KF, being proximal muscles and more degenerative, showed no change in function at 9 months after MTT. The KE, being proximal and anti-gravitational, were most degenerative before MTT. They showed no statistically significant change in force at three months after MTT but showed decreases at 6 and 9 months after MTT. At 9 months after MTT, 23% of the 60 KE

examined showed a mean increase of 65% in force; 22% showed no change; and 55% showed a mean decrease of 24% in force. When results of all muscle groups (AF, KF, KE) were pooled, there was no change in force at 3, 6, or 9 months after MTT versus before MTT according to the Wilcoxon signed rank test. The ambulatory subjects showed more muscle improvement than the nonambulatory ones at various times after MTT. Statistically significant progressive increase in force in the AF and arrest of weakening in the KF and KE were observed in the ambulatory subjects as early as 3 months and continued up to 9 months after MTT.

The results indicated that (1) MTT was safe; (2) MTT improved muscle function in DMD: 88% of the AF, 49% of the KF, and 45% of the KE showed either increase in strength or did not show continuous loss of strength 9 months after MTT; (3) LBT significantly strengthened the lower bodies in one-third of the 32 DMD boys, improving their locomotive ability and quality of life. The behavioral improvement subsided for insufficient dosage of myoblasts and continual upper body weakening; (4) the dosage used was more effective in the AF than in the KF, and was least effective in the KE; (5) more than five billion myoblasts were necessary to strengthen both lower limbs of a DMD boy between 6 and 14 years of age; (6) the more degenerated proximal muscles would need more myoblasts per unit muscle volume than the distal muscles for MTT to be effective; (7) MTT was more effective in the younger, ambulatory subjects than in the older, nonambulatory subjects; (8) cyclosporine was not responsible for the functional improvement, since muscle function continued to improve 9 months after MTT despite cyclosporine withdrawal at 6 months after MTT; (9) cyclosporine immunosuppression permits donor cell survival and development, without overt rejection symptoms, when properly managed; (10) myoblasts from frozen reserves were as effective as those from fresh muscle biopsies; (11) fifteen billion myoblasts could be cultured from a 1-g muscle biopsy; and (12) billions of cultured myoblasts could be injected into subjects without tumor formation (Law et al. 1992).

Whole Body Treatment (WBT): Phase II and III Trials

Rationale

One of the conclusions of the LBT study was that the dosage used was more effective in the distal muscles than in the proximal muscles. MTT might be more effective in the more degenerative proximal muscles if the number of myoblasts per unit muscle volume was increased. Furthermore, the LBT study indicated that more than five billion myoblasts were necessary to strengthen both lower limbs of a DMD boy between 6 and 14 years of age (Law et al. 1992).

Whereas some of the subjects showed muscle strengthening in both distal and proximal muscles of their lower limbs, their upper body muscles continued to degenerate, with drastic loss of function and balance. The latter prompted us to initiate the administration of myoblasts to muscles in the upper bodies of DMD subjects, and, if safe, to further readminister myoblasts to muscles in their lower bodies.

Dose Escalation

The first administration of eight million myoblasts, starting in February 1990, into a small foot muscle of 11 subjects did not generate any graft-versus-host reaction. The second administration of five billion myoblasts into the lower body of 32 subjects, which represented a dose increase of 625 folds, again did not produce any adverse effect. The last dose was increased 2.5 times, to 12.5 billion myoblasts injected into the upper body. If this dose was found to be safe within 2–3 months after this upper body treatment (UBT), it would be further assessed for accumulative safety by readministering an additional 12.5 billion myoblasts to muscles of the lower body. Thus, this WBT protocol examined a maximum dose of 25 billion myoblasts to be widely distributed in the whole body of each DMD subject (Law et al. 1993).

Objective

The objective of this project was to determine the safety and efficacy of injecting 25 billion cultured myoblasts into 64 large dystrophic muscles of DMD boys over a 9-month period. The study tested (1) whether widely distributed multiple intramuscular injections with large numbers of cells delivered on two separate occasions all over the body were safe; (2) whether MTT was effective in improving, or halting the deterioration of, muscle function in biceps brachii, triceps, knee extensors, and ankle plantar flexors; (3) whether MTT was effective in improving, or halting the deterioration of, muscle structure in the biceps brachii and quadriceps; (4) whether MTT would induce production of dystrophin in the myoblast-injected biceps brachii and quadriceps; and (5) whether MTT in the upper body would improve pulmonary function.

The goal of this WBT clinical trial was to determine whether MTT could be a safe and effective treatment program against muscular weakness in DMD. If MTT was effective, strengthening the arm muscles would improve motor control; strengthening the back musculature would improve balance; strengthening the lower limb muscles would improve locomotor ability; and strengthening the accessory respiratory muscles would improve pulmonary function and prevent premature death.

Study Design

Thirty-two DMD boys, aged 6–16 each underwent two procedures of MTT: an UBT in which 28 muscles were injected with 12.5 billion myoblasts, and a LBT in which an additional 12.5 billion myoblasts were injected in another 36 muscles (Law et al. 1993). Subjects were selected into two equal groups, ambulatory and non-ambulatory. Half of each group was randomized to receive early UBT or LBT treatment. The late treatment group served as control for the early treatment group. Subjects receiving early UBT received sham injections in one randomly selected biceps brachii; subjects receiving early LBT received sham injections in one randomly selected quadriceps. The contralateral biceps or quadriceps's were myoblast injected. In addition, each boy received injections in major muscle groups of the neck, back, upper limbs, shoulders, abdomen, and lower limbs bilaterally. The immunosuppressant cyclosporine was administered from 5 days before until 3 months after each MTT.

At 3 months and at 3 days before each MTT, and at 3, 6, and 9 months after each MTT, the maximum voluntary contractions of the biceps brachii, triceps, quadriceps, and ankle plantar flexors were measured with Kin-Com dynamometry. Muscles of MTT-2 were examined at 12, 9, and 6 months before MTT to determine the progression of the disease. Pre-MTT data yielded natural history controls. Comparison of the myoblast-injected versus the placebo muscles would determine if MTT was beneficial.

At 9 months after MTT-1, biopsies were obtained from the biceps or quadriceps. The presence of dystrophin in immunocytochemical and Western immunoblot assays substantiated graft survival and biochemical improvement. Reduction of dystrophic characteristics such as central nucleation, fiber splitting, phagocytic necrosis, and infiltration of fat and connective tissues were indicative of structural improvement. Correlation of biochemical, structural, and functional data would determine the efficacy of MTT, if any. Absence of adverse reactions would substantiate MTT safety.

UBT/LBT Randomization

Sixteen ambulatory subjects were randomly assigned to receive either early UBT treatment or early LBT treatment, with the constraint that an equal number of subjects (8) be assigned to each group. Similar randomization for the 16 non-ambulatory subjects was also be instituted.

Myoblast/Placebo Randomization

The randomization design (Law et al. 1995) also included a randomized, double-blind study of one muscle in MTT-1. Subjects assigned to early UBT had one biceps brachii selected for myoblast injections with the contralateral biceps brachii receiving sham injections. Subjects receiving early LBT had one quadriceps selected for myoblast injections with the contralateral quadriceps receiving sham injections. Within each of these two treatment groups, equal numbers of subjects received myoblast injections on the right side and sham injections on the left side, and vice versa.

The biceps brachii were solely responsible for flexion of the forearm; the quadriceps were solely responsible for extension of the leg. As proximal muscles, they were more involved in the degenerative process of dystrophy than distal muscles. The randomization code was applied by an in-house person as subjects were entered into the study. He advised the administering surgeon of the designation of the MTT and placebo arms/legs. These two individuals were the only ones who knew which side was injected with myoblasts and which side was injected with saline. Neither the patient, nor his family and personnel evaluating MTT efficacy were made aware of which arm/leg received myoblasts and/or which arm/leg served as control. The surgical suite was configured so that those outside the suite could not see the injections.

Donors

The donors were male volunteers, in good general health, and between the ages of 8 and 22 (Law et al. 1995). They were certified by physicians as being in good health, not having a CK level of more than twice the upper limit of normal, and testing

negative for human immunodeficiency virus (HIV), hepatitis B surface antigen (HBSAg), hepatitis C (HCV), syphilis (RPR), and cytomegalovirus (CMV). Donors would be excluded if they had any chronic, infectious diseases or were allergic to the local anesthetic. Conformity to the selection criteria were documented by the clinical monitor. Informed consents were obtained from the donors or from the parents of minors after thorough discussion of the procedures and risks.

Myoblast Preparation

Two months before MTT, the donor came to the hospital outpatient clinic to donate muscle biopsy. Three days before each biopsy, a 22 gauge spinal needle (2 inches long) were inserted momentarily without injection several times into the future biopsy site (about 1 inch diameter) of the rectus femoris muscle of the donor to induce satellite cell proliferation. These needle insertions, done under local anesthetic using lidocaine, were completed in about 30 s and would cause short-term (< 5 min) pain. The area would bruise for a few hours. Three days later, under local anesthesia and in a sterile field, about 2 g of muscle were surgically removed from the site of injury using an open biopsy technique (Law et al. 1995). The biopsied muscle was immediately dissociated. Complete dissociation of 2 g of fresh skeletal muscle occurred after 45 min of stirring with three changes of collagenase solution, alternated with three changes of neutralizing medium. Cells were then placed in fresh growth medium. Myoblasts were fed with fresh growth medium every 2 days and incubated in 7% CO_2 at 37 °C as previously described.

Myoblasts were harvested for injection when they filled 250 roller bottles, each containing about 50 million myoblasts on a 900 cm^2 surface as determined with a hemocytometer. Representative samples of myoblasts were tested for their ability to divide, fuse, and form myotubes and immature myofibers that could contract spontaneously in vitro. Donor myoblasts were detached from each bottle with trypsin solution. An equal volume of neutralizing medium was then added to stop the action of trypsin after 5 min of incubation. Cell suspension from 250 roller bottles was pooled for each harvest. Myoblasts were centrifuged, rinsed three times, and resuspended in 312.5 ml of a solution containing 5% host serum in injection saline (Sigma). The procedure of harvesting was timed in each case to relay immediately to the procedure of myoblast transfer itself. The culture procedure took about 6 weeks to obtain the 25 billion myoblasts from a 2 g muscle biopsy. It took ten workers 4 h to complete harvesting 250 roller bottles of myoblasts under five laminar flow sterile hoods (Law et al. 1995).

The 25 Billion Myoblast WBT Protocol

Under FDA purview, 1 infantile facioscapulohumeral dystrophy and 40 DMD boys aged 6–16 received WBT in 36 months with no adverse reaction. Subjects took oral cyclosporine for 3 months after each MTT. Nine months after MTT immunocytochemical evidence of dystrophin were demonstrated in 18 of the 20 DMD subjects biopsied. Dystrophin positive sections showed less dystrophic characteristics than dystrophin-negative ones. Forced vital capacity increased by 33.3% and maximum voluntary ventilation increased by 28% at 12 months after UBT (Law et al. 1994b, 1995, 1996, 1997b, c, d, 1998, 65).

Plantar flexion showed an increase of 45% in maximum isometric contraction force in 12 months in the DMD subjects when compared to the natural deterioration. Behavioral improvements in running, balancing, climbing stairs, and playing ball were noted (Law et al. 1994b, 1995, 1996, 1997b, c, d). Notable was a 16-year-old DMD subject who continued to walk without assistance and capable of driving an automobile by himself.

The 50 Billion Myoblast WBT Protocol

The most effective design involved a one-time injection of fifty billion myoblasts into 80–82 muscles with 179 skin punctures, approved by the FDA for subjects with DMD, Becker MD, and limb-girdle MD (Law et al. 1997a, b, c, 1998). Over 200 subjects who underwent this protocol had experienced no serious or permanent adverse reaction. None of the subjects died within 2 years after MTT. The demonstrated efficacy included:

- Correction of gene defect with dystrophin production induced by MTT.
- 70% more myofibers and histological improvement at 9 months after MTT.
- 123% increase in maximal contractile force at 18 months after MTT.
- 39% decrease in serum CPK at 12 months after MTT.
- 19% increase in forced vital capacity at 9 months after MTT.
- Clinical improvement in 75% of all subjects participated.
- Life prolongation up to 45 years of age with many subjects in their 30s.

WBT Significance

During Phase II and Phase III clinical trials from 1994 to 1999, FDA allowed CTRF charging up to USD150,000 per MTT procedure after annual review of progress. On October 16, 1998, FDA designated CTRF's MTT program on Fast Track, once again confirming its safety and efficacy. The charge allowed by the FDA was often paid by governments or health agencies of the USA, Russia, West Germany, Hungary, Brazil, Mexico, and Korea for their citizens.

According to the late Leon Charash, former Director of the Medical Committee of MDA, it was jealousy, greed, and insecurity that drove the MDA to eventually derail the CTRF's MTT programs (Law 2017).

Heart Muscle Degeneration

Heart muscle degeneration is the leading cause of debilitation and death in humans. Atherosclerosis, ischemic cardiomyopathy, and heart failure are genetic predisposed (Beutner et al. 2011). These are multifactorial and polygenic diseases with significant polymorphism. It will be an insurmountable task to identify the various gene defects and to design molecular gene therapies toward treatment, not to mention that such designs do not replenish myocardial cells that had degenerated previously, without which the damaged myocardium cannot regain its function.

The primary cause of ischemic cardiomyopathy is partial or complete obliteration of the coronary artery, often precipitating in acute myocardial infarction (MI). Long-term ischemia leads to ventricular cell death and myocardial necrosis. Although medication, trans-myocardial revascularization (TMR), and coronary artery bypass grafting (CABG) can increase local blood supply and reduce short-term mortality and morbidity, most patients continue suffering decreased ventricular function and chronic heart failure.

Severe Myocardial Infarction

Severe myocardial infarction conveys serious complications such as ventricular aneurysm, wall thinning, and rupture with fatal consequences. Prognosis for these severe heart failure patients is 3–6 months of life despite CABG. Heart transplantation has been the gold standard for patients with end-stage heart failure, but donor heart nonavailability and lifelong immunosuppression dictate its limited usage. It is urgent to explore new therapeutic measures to increase the quality of life and life span of these patients.

Chronic ischemia after AMI causes loss of cardiomyocytes, contractile filaments, and ventricular contractility. Regeneration attempt by surviving cardiomyocytes consists of undergoing at most four mitotic divisions because the telomeric DNA repeats (Ishikawa et al. 1993) in these terminally differentiated cells are minimal. Such attempt cannot regenerate enough cardiomyocytes to produce the necessary quantity of contractile filaments such as myosin, actin, troponin, and tropomyosin to sustain normal heart contractility. The degenerative heart also transmits biochemical signals to recruit stem cells from the stroma and from the bone marrow to repair the muscle damage. Due to the significant increase in fibroblast growth factor release after infarction, much of the recruited stem cells differentiate to become fibroblasts instead of cardiomyocytes, forming scars and not contractile filaments.

The damaged myocardium needs replenishment of live, genetically normal, myogenic cells to deposit contractile filaments to regain heart function, preferably before fibroblast infiltration. This is when allogeneic myoblast transplantation (AMT), formerly known also as MTT or heart cell therapy, has an advantage over all other cell types (Law et al. 2004a, b). Considering dystrophic and diabetic cardiomyopathies as hereditary, and ischemic cardiomyopathy as genetically predisposed, AMT was designed to genetically repair hereditary degenerative cells and to replenish degenerated cells with live ones.

Proof-of-Concept for AMT

Myoblasts are differentiated cells destined to become muscles. Unlike cardiomyocytes, myoblasts possess long telomere DNA subunits and can undergo 50 mitotic divisions without loss of myogenic capacity (Law et al. 2004a, b) or developing into tumor (Di Donna et al. 2003). The transition from animal

experimentation (Koh et al. 1993; Taylor et al. 1998) into cardiac clinical trials was largely based on previous demonstration of significant safety and efficacy in the treatment of Duchenne muscular dystrophy in FDA approved Phase II and Phase III clinical trials using allogeneic myoblast allografts (Law 2004; Law et al. 1998, 2019).

Law et al. reported the pioneering study of implanting human myoblasts into the porcine hearts with endovascular catheter of the NOGA system (Law et al. 2000). The mechanisms by which allogeneic human myoblasts survived, developed, and functioned with the use of cyclosporine immunosuppression were examined through open chest endomyocardial injections of cultured skeletal myoblasts into infarcted porcine myocardium. Three mechanisms were elucidated as proof-of-concept using genetic markers to label the nuclei of the donor myoblasts (Law et al. 1992, 2004a, b). Under the influence of hormones and slow contractile activity of the heart, donor myoblasts fused among themselves to form new cardiomyocytes, depositing contractile filaments to improve heart contractility. Others fused with the host cardiomyocytes through natural cell fusion, spontaneously transferring their nuclei into host cardiomyocytes to impart myogenic regeneration. Still others fused to form myotubes that eventually developed into immature skeletal myofibers containing satellite cells. New production of contractile filaments augmented heart contractility (Law et al. 1992, 2004a, b).

AMT Is Safe and Efficacious with MI Patients

In 2004, Law et al. reported the world's first two cases of using allogeneic myoblast transplantation (AMT), cyclosporine immunosuppression, and CABG for MI (Law et al. 2004b, 2006). Results demonstrated that the left ventricular ejection fraction (LVEF), viability, motility, myocardial perfusion, and ventricular wall thickness were significantly improved. The end-diastolic and end-systolic blood volumes were increased without significant arrhythmia. At 12 months after implantation, LVEF was increased by about 40%, and perfusion capacity increased by 38% (Law et al. 2004b, 2006).

More than 300 cases of autologous myoblast transplantation internationally have showed that it was safe and efficacious in treating ischemic cardiomyopathies and heart failure. Approximately 200 cases were injected after thoracotomy and 100 cases were injected with endovascular catheters (Law et al. 2006, 2019). Further advance with myoblast clinical trials had been distracted for 17 years by Piero Anversa's misconduct in cardiac stem cell research in Harvard University until 10/14/2018.

AMT Is Safe and Efficacious with End-Stage HF Subjects

Recently, AMT, cyclosporine immunosuppression, and CABG were used to treat end-stage HF subjects without hope of obtaining a heart transplant (Li et al. 2021). It was demonstrated to have sustained the life spans to 2 years postoperatively on ten

end-stage HF patients having 2.21-mm mean thinnest wall thickness and ventricular aneurysms, with significant improvement in LVEF, NYHA cardiac function, viability, and quality of life.

Regulatory

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Xinxiang Medical College to obtain preliminary safety and efficacy information to justify future clinical trials of AMT. This study was registered in the Chinese Clinical Trial Registry numbered **ChiCTR2000039590**.

Case Selection

A total of ten heart failure subjects suffering severe MI with ventricular aneurysm in the Cardiothoracic Surgery Department of the Third Affiliated Hospital of Xinxiang Medical College from February 2016 to March 2018 were selected. Subject profiles are shown in Table 1.

Table 1 General information of subjects. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Gender	Age	Combined disease	Infarct area	Abnormal movement (Y/N)	Bypass vessels
Wang	F	53	History of cerebral hemorrhage, hypertension	Anterior and interval wall	N	LAD, LCX, D1
Huang	M	31	None	Anterior wall near apex	N	LAD, LCX
Cui	M	72	Membranous nephropathy, diabetes	Anterior, interval, and rear wall.	N	LAD, LCX
Ren	M	62	Hypertension	Anterior wall near apex	N	LAD, LCX
Song	M	60	Hypertension, diabetes	Anterior, interval, and inferior wall.	N	LAD, LCX, D1
Zhou	M	53	None	Anterior wall near apex	N	LAD
Wang	M	68	None	Anterior wall near apex	N	LAD
Che	M	60	Hypertension	Anterior wall near apex	N	LAD, LCX, LCA
Du	M	60	None	Anterior wall near apex	N	LAD, LCX
Zhang	M	64	Hypertension, history of cerebral, hemorrhage, and diabetes	Anterior wall near apex	Y	LAD, LCX

Patient Selection

After meeting Inclusion/Exclusion criteria and signing Patient Informed Consents, ten HF subjects having mean thinnest wall thickness of 2.21 ± 0.55 mm and ventricular aneurysms were admitted under intensive care. Each subject took daily cyclosporine for 3 weeks. On the third day of cyclosporine administration, approximately one billion myoblasts were implanted through 20 injections into the infarcted myocardium following CABG.

Manufacture of Allogeneic Human Myoblasts

Donors. Upon approval of the Institutional Review Board (IRB) of the Cell Therapy Institute and the signing of the Donor Informed Consents, muscle donors were admitted after meeting the Inclusion and Exclusion criteria. They were male volunteers between the ages of 16 and 36. They were certified by a physician as being in good health, having normal levels of aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LD), and tested negative for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), syphilis (RPR), and cytomegalovirus (CMV). They also received the following tests: Chem 24, CBC, and physical examination with normal results. Donors were excluded if they had any chronic or infectious diseases, and if were allergic to the local anesthetic lidocaine.

Muscle Biopsy. About 2 g of muscle were removed from the quadriceps muscle using an open biopsy technique under local anesthetic (lidocaine patch) in a sterile field of a surgical suite of a hospital. The donor site was sutured and bandaged. No prophylactic antibiotic was used. The donor was discharged after recovery from the surgical procedure to be followed by his physician if infection occurred.

Preparation of Myoblasts. Biopsy specimen obtained was processed immediately using sterile techniques meeting CFDA approved GMP standards. Myoblasts were cultured in growth medium and incubated at 37 °C and in 7% CO_2 as previously described (Law et al. 1992; Law 1995). Myoblasts were frozen at different stages so the time allotted for culturing could be coordinated with a scheduled transplant. The number of frozen cells and the number of samples were documented. One test vial was reserved in liquid nitrogen for each biopsy. Random samples of the myoblasts were tested for their ability to divide, fuse, and form myotubes (Law et al. 1992). Lot release testing consisted of sterility, endotoxin, mycoplasma, and testing for myoblast identity, purity, potency, viability, and cell count on a pooled sample prior to transplant meeting quality control standards (Law et al. 1992, 2008; Law 1995). A retain sample of myoblasts was reserved from each transplant.

Cyclosporine Immunosuppression

Each subject took daily oral doses of 5 mg/kg body weight of cyclosporine for 3 weeks with weaning using half doses after the second week to suppress rejection of the allografts. Since myoblast fusion completed within 1 week after transplantation (Law et al. 1994a; Haider et al. 2004c), and since myotubes and mature myofibers did not express MHC class 1 surface antigens (Law 1994; Law et al. 1994a), it was not necessary to administer lifelong immunosuppression as in heart transplants (Law et al. 2019). Clinical evaluation was performed using a self-contrast method.

Clinical Research Procedures

All patients were given CABG and conventional medication for symptomatic control of blood pressure, blood lipid, blood glucose, anticoagulation, coronary expansion, cardiac strengthening, and diuretics. Traditional aneurysm resection was not used in this study.

AMT

AMT was performed following distal anastomosis of the coronary artery bypass surgery on the third day of cyclosporine administration. Approximately 1 billion myoblasts (at 10^8 cells /mL) were injected with 20 injections placed 1 cm apart along the inside border of the infarction. For each injection, about 50 million myoblasts were carefully deposited in a centripetal diagonal track (< 5 mm) as the needle was slowly withdrawn out of the inner border of the infarcted myocardium.

Data Analysis and Interpretation

Statistical Analyses

Data were analyzed with one-way analysis of variance (ANOVA) using SPSS19.0 software. $P < 0.05$ indicated significant statistical differences.

Safety Assessment

Adverse Reaction Assessment

All ten subjects successfully underwent treatment without malignant arrhythmia, chills, fever, allergic reactions, vomiting, viral infection, or other adverse reactions. Throughout the course of the entire study, two subjects exhibited early postoperative occasional ventricular premature beats. Another two subjects demonstrated atrial premature beats. Considered related to electrolyte disorders and myocardial damage of the primary disease, they were converted to sinus after symptomatic treatment. No malignant arrhythmia and no deaths occurred, and there was no virus infection after treatment. There were no statistically significant differences between the means (±SD) in the leukocyte count, neutrophil count, hemoglobin, red blood cell count, and platelet count before versus at 6 months, and at 2 years after treatment ($P > 0.05$) (Table 2).

Table 2 Blood cell test results before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	2 years postoperative	P
Leukocyte count	7.59 ± 3.19	8.29 ± 2.45	8.18 ± 1.95	0.808
Neutrophil count	3.25 ± 0.88	3.35 ± 0.55	3.24 ± 0.75	0.934
Hemoglobin	147 ± 9.94	144.8 ± 9.94	142.8 ± 9.42	0.635
Red cell count	4.41 ± 0.63	3.95 ± 0.60	4.04 ± 0.77	0.279
Platelet count	213.4 ± 48.95	245.60 ± 52.15	236.10 ± 40.36	0.312

Table 3 Liver function indexes before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	2 years postoperative	P
Total bilirubin (μmol/L)	18.02 ± 10.6	20.31 ± 10.17	15.46 ± 5.11	0.491
Albumin (g/L)	41.98 ± 4.46	42.76 ± 3.74	44.25 ± 2.52	0.138
Alanine aminotransferase (U/L)	43.80 ± 34.77	29.80 ± 18.67	24.20 ± 5.98	0.166
Aspartate aminotransferase (U/L)	30.25 ± 7.29	30.5 ± 7.8	26.13 ± 7.74	0.161

Table 4 Renal function indexes before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	2 years postoperative	P
Serum creatinine (μmol/L)	76.90 ± 16.91	112.40 ± 81.32	82.80 ± 14.77	0.236
Urea nitrogen (g/L)	4.11 ± 1.05	4.78 ± 0.98	4.03 ± 1.05	0.776

Table 5 Electrolyte levels before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	1 year postoperative	P
K^+	3.98 ± 0.35	4.08 ± 0.42	4.01 ± 0.35	0.831
Na^+	140.6 ± 2.98	142.1 ± 3.31	142.1 ± 2.80	0.456
Cl^-	102.30 ± 2.83	103.3 ± 3.40	103.9 ± 5.52	0.680
Ca^{2+}	2.17 ± 0.09	2.29 ± 0.10	2.26 ± 0.13	0.075

There was no statistically significant difference between the mean levels (± SD) of total bilirubin, albumin, alanine aminotransferase, and aspartate aminotransferase before versus at 6 months, and at 2 years after treatment ($P > 0.05$) (Table 3). There was no statistically significant difference between the mean levels (± SD) of serum creatinine or urea nitrogen before versus at 6 months, and at 2 years after treatment ($P > 0.05$) (Table 4). There was no statistically significant difference in the mean levels (±SD) of serum K^+, Na^+, Cl^-, and Ca^{2+} before treatment versus at 6 months and at 2 years after treatment ($P > 0.05$) (Table 5). There was no statistically significant difference in the mean levels (± SD) of prothrombin time, fibrinogen, and partial prothrombin time before versus after treatment ($P > 0.05$) (Table 6).

Efficacy Assessment

Objective Evaluation

Cardiac ECT demonstrated significant improvement in myocardial perfusion and in viability of ventricular myocardium at 6 months and at 2 years after surgery (Fig. 1). The mean LVEFs were increased by 20.1% at 6 months after treatment, and by 19.3% at 2 years after treatment as compared to the control mean before treatment.

Table 6 Blood coagulation indexes before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	2 years postoperative	P
Prothrombin time(s)	13.18 ± 0.50	13.30 ± 0.54	4.01 ± 0.35	0.951
Fibrinogen (g/L)	3.13 ± 0.17	3.26 ± 0.17	2.88 ± 0.20	0.341
Activated partial Thromboplastin time(s)	29.55 ± 1.13	30.94 ± 0.98	32.63 ± 1.22	0.169

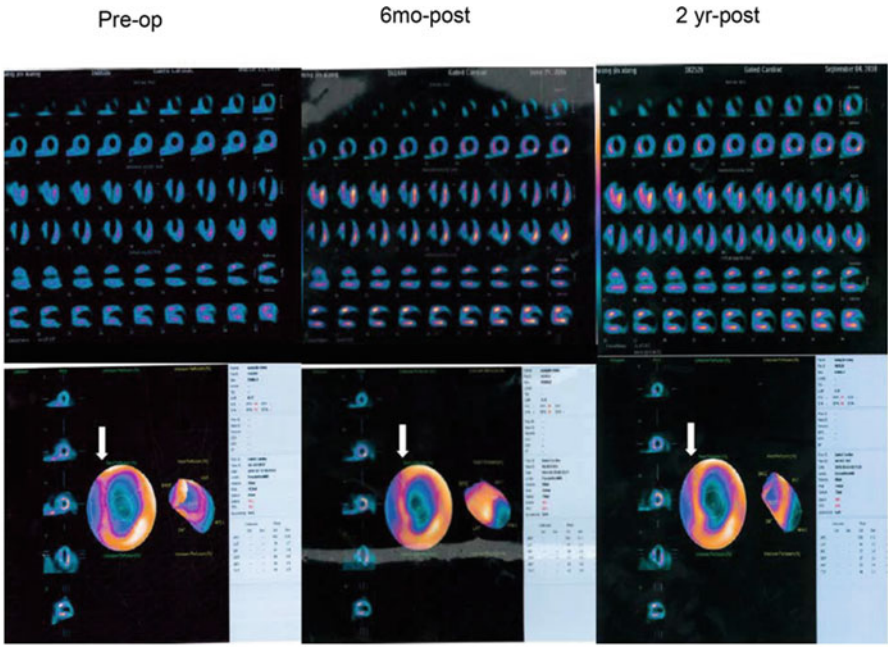

Fig. 1 Representative ECT indicated significant increase in viability (Arrow) of ventricular myocardium at 6 months and at 2 years after surgery. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

These mean increases were of statistical significance at $P < 0.05$ (Figs. 1 and 2a; Table 7). There was no significant difference (Table 7) in the mean levels (±SD) of LVDd (Fig. 2b) and CO (Fig. 2c) before versus after treatment ($P > 0.05$). Although the mean difference in CO before versus after treatment was of statistical insignificance, the postoperative means were all higher than before treatment. Consistently, the mean postoperative lengths of LVDd were lower than that before treatment. MR imaging showed no significant difference in the mean levels (±SD) of ventricular aneurysm area and wall thickness before versus after treatment ($P > 0.05$) (Table 8) though individual variations did demonstrate significant increases in wall thickness (Fig. 3).

Table 7 Cardiac function before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	2 years postoperative	P
LVEF	39.3 ± 8.76	47.2 ± 5.44	46.9 ± 5.69	0.029
LVDd	60.3 ± 9.86	57.3 ± 8.84	54.8 ± 7.24	0.381
CO	5.18 ± 1.12	6.02 ± 1.18	6.07 ± 0.94	0.351

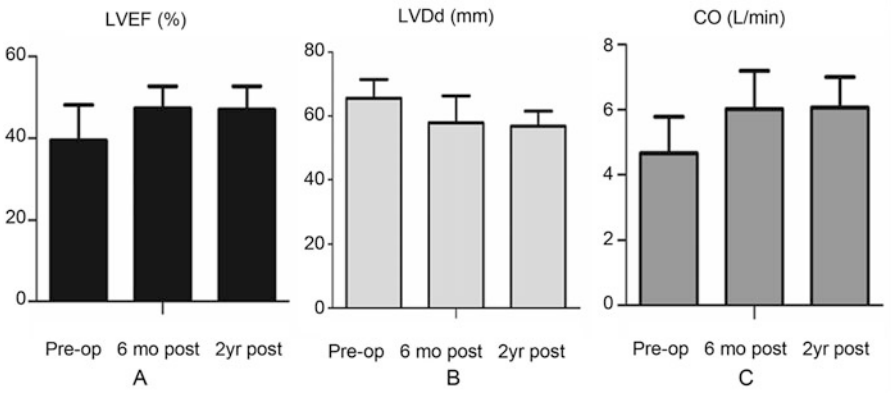

Fig. 2 (**a**) LVEF showed significant increases at 6 months and at 2 years after treatment. There was no statistically significant difference in mean LVDd (**b**) and mean CO (**c**) before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

Table 8 Ventricular aneurysm area and thinnest wall thickness before versus after treatment. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

	Preoperative	6 months postoperative	2 years postoperative	P
Area, mm^2	460.1 ± 143.14	394 ± 150.93	422.7 ± 426.31	0.864
Thickness, mm	2.21 ± 0.55	2.32 ± 0.51	2.49 ± 0.70	0.565

Subjective Evaluation

Postoperative clinical improvement included significantly fewer episodes of angina pectoris, chest tightness and shortness of breath after activity, nighttime sit-up breathing, and increase in appetite and urine output. The 6MWT distance increased steadily. The results depicted essentially derived from allogeneic myoblast culture and transplantation (Fig. 4), in addition to CABG and cyclosporine immunosuppression.

Understanding and Debating the Study Outcomes

For the first time, AMT in adjunct use with CABG and cyclosporine was demonstrated to be safe and efficacious in sustaining the life span of ten end-stage heart failure patients, who suffered myocardial infarction with ventricular aneurysm, for up to 2 years, with significant improvement in LVEF (Table 7), NYHA cardiac function (**Central Illustration**; Fig. 5) and quality of life.

Central Illustration: NYHA grading before versus after treatment

	Gender	Age	Pre-op	6 months post-op	2 years post-op
Wang	F	53	III	II	II
Huang	M	31	III	I	I
Cui	M	72	III	II	I
Ren	M	62	III	I	I
Song	M	60	IV	II	II
Zhou	M	53	III	II	I
Wang	M	68	II	I	I
Che	M	60	III	I	I
Du	M	60	IV	II	II
Zhang	M	64	III	II	I

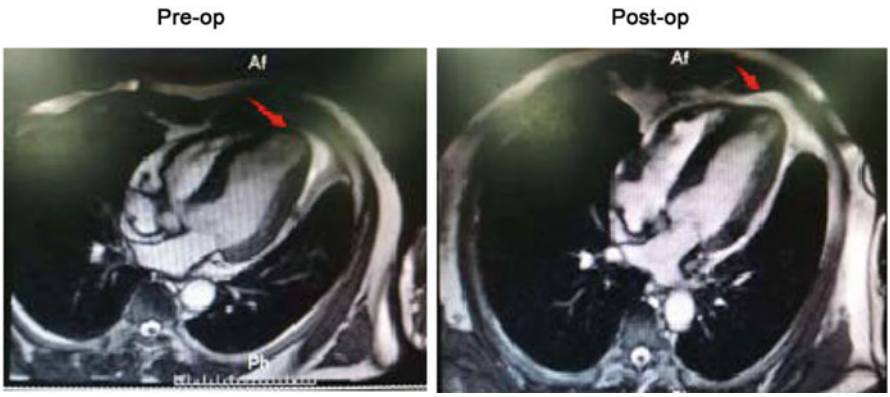

Fig. 3 MRI indicated the increase in wall thickness after treatment (red arrows). (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

It was the original design to define a treatment modality that could 100% sustain the survival of the severe heart failure patients having no more than 6 months of life expectance, and such design was demonstrated to be successful. The statistically significant increases in the mean LVEF by 20.1% at 6 months after treatment, and by 19.3% at 2 years after treatment compared favorably to those reported by all other studies using autologous myoblasts (Menasché et al. 2001a, b, 2003, 2008; Ghostine et al. 2002; Herreros et al. 2003; Siminiak et al. 2004, 2005; Dib et al. 2005; Fernandes et al. 2006; Gavira et al. 2006; Steendijk et al. 2006; Menasché 2007; Duckers et al. 2011; Povsic et al. 2011; Sawa et al. 2015). As the trend of improvement indicated, statistically significant difference in LVDd and CO before versus after treatment would have become apparent if more subjects were enrolled.

The ultimate demonstration of AMT efficacy by itself should be from randomized, double-blinded studies involving more subjects of dystrophic cardiomyopathies, diabetic cardiomyopathy, or dilated cardiomyopathies that would not necessitate CABG. This study showed that white blood cell count, neutrophil

Fig. 4 Schematic diagram of myoblast transplantation. (**a**) Myoblast culture of 20 roller bottles produced approximately ten billion cells; (**b**) Myoblasts were produced under GMP condition; (**c**) Myoblasts of >90% purity according to desmin immunostain; (**d**) Approximately 10 billion myoblasts were injected into the myocardial infarcted ventricle. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

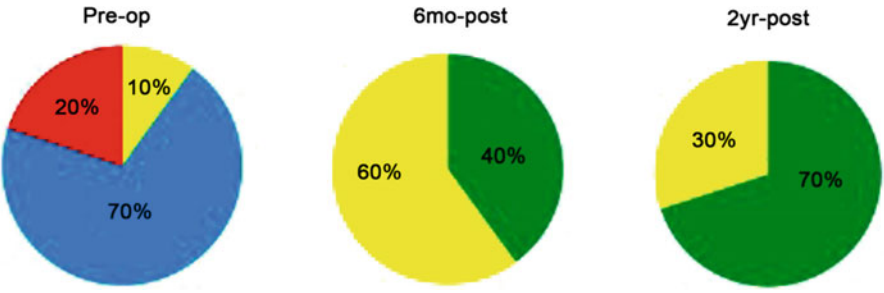

Fig. 5 NYHA cardiac function improved by two grades at 2 years postoperatively. Green indicates Grade I, yellow II, blue III, and red IV. (Reproduced with permission from Li et al. *Open Journal of Regenerative Medicine* 2021; 10: 1–18. © Scientific Research Publishing)

count, total bilirubin, alanine aminotransferase, aspartate aminotransferase, total red blood cells, hemoglobin, platelets, total protein, renal function, fibrinogen, prothrombin time, and partial prothrombin time before versus after treatment of

myoblasts were not different ($P > 0.05$). On repeated follow-ups after the operation, there were no deaths, malignant arrhythmias, or viral infections. The results demonstrated that allogeneic myoblast transplantation had no significant impact on various important indicators of the human body, and there was no serious adverse reaction. Although occasional ventricular premature beats occurred in four subjects after CABG, all symptomatic treatments were traced to the sinus and were related to electrolyte disturbance, myocardial damage, and the primary disease. None of the subjects experienced malignant arrhythmia or ventricular tachycardia.

AMT represented the earliest human cell therapy for heart disease (Law et al. 2000). Without significant knowledge of myoblast manufacture, quality control, and cell transplant techniques, many clinicians rushed into clinical studies. Menasche et al. first reported feasibility and safety data on ischemic heart failure patients, implanting 650 million to one billion autologous impure myoblast cells with overly large number of injections into the infarctions during CABG (Menasché et al. 2001a, b). Follow-up studies showed that patients had significantly improved left ventricular function and NYHA function level, decreased ventricular remodeling, and increased myocardial tolerance confirmed by histology. However, four patients had delayed episodes of sustained left ventricular tachycardia (Menasché et al. 2001b). During this period, similar reports continued to appear around the world with mixed results.

In most of the reports, myoblast purity was determined using $CD56^{+}$, an antibody that reacts with fibroblasts, neurons, and myoblasts indiscriminately. A common pitfall of myoblast culture is fibroblast contamination. Since myoblast doubling time is about 21 h and fibroblast doubling time is about 15 h, fibroblast growth often overtakes the myoblast culture (Law et al. 1992; Law 1995). Without published documentation of quality controls, authors of these studies were implanting very impure myoblasts. Fibroblasts produce scars but not contractile filaments. These scars created numerous barriers to electric coupling and rhythmic synchronization of ventricular contraction. This pitfall, together with the physical trauma induced by overly large number of injections, were largely responsible for the malignant arrhythmia and tachycardia reported in some early patients, not to mention the gross GMP noncompliance in quality control of myoblast identity, viability, purity, quantity, potency, no mycoplasma, no endotoxins, and no bacteria (Ghostine et al. 2002; Menasché et al. 2003, 2008; Herreros et al. 2003; Siminiak et al. 2004, 2005; Dib et al. 2005; Fernandes et al. 2006; Gavira et al. 2006; Steendijk et al. 2006; Menasché 2007; Duckers et al. 2011; Povsic et al. 2011; Sawa et al. 2015). The latter explained cell death of up to 90% that affected the safety and efficacy of myoblast transplantation (Suzuki et al. 2004). Clinical studies confirmed that CABG with transplantation of impure and nonviable myoblasts produced ventricular tachycardia (Fernandes et al. 2006). The intercalated disc, the basic unit for the transmission and synchronization of electrical activity and mechanical function between adjacent cardiac fibers were not found, and its absence constituted a risk of malignant arrhythmia after transplantation with substandard myoblasts (Ferreira-Cornwell et al. 2002). Therefore, the safety and efficacy of myoblast transplantation depend largely on high quality control of myoblast production and the technique of implantation.

Heartbeat is myogenic in origin and is initiated by pacemaker activity in the sinoatrial node. As depolarization sweeps through the atrioventricular node, the depolarization excites the Purkinje fibers of the bundle of His, which, in turn, signals the ventricles to contract rhythmically. Heart function would be impaired if the rhythmic action potentials do not synchronize the fiber contractions. In the regenerative heart with AMT, excitation of the newly formed and heterokaryotic cardiomyocytes remained unchanged because there was little change in gap junctions for current flow. However, where new skeletal myofibers (Law et al. 2004a, b, 2006; Pagani et al. 2003) were formed, presumably at the inner border of the infarction, such heterogeneity might create aberrant electric activities such as arrhythmia, especially when earlier studies reported absence of gap junction protein connexin 43, a marker protein responsible for electrical coupling between cardiomyocytes (Tolmachov et al. 2006).

Conceptually, myoblast transplantation should not cause arrhythmia if the well-researched standard operation procedures were followed. The thresholds of excitation for cardiac and skeletal myofibers are similar, i.e., between +40 mV and +50 mV depolarization. Whereas the cardiomyocyte action potential is triggered with an increase in Ca^{2+} conductance into the cell, the skeletal myofiber action potential is triggered with an increase of Na^{+} conductance. As Ca^{2+} has a greater ionic size than Na^{+} and thus lower ionic mobility, the action potential of cardiomyocytes has a longer duration (~250 ms) than that of skeletal myofiber (~1.5 ms). This duration difference is advantageous because the same myocardial depolarization can simultaneously and synchronously stimulate the cardiac and skeletal myofibers through direct excitation contraction coupling. Since the action potentials of skeletal myofibers are of short duration, they will merge into the longer compound action potential of the heart. The skeletal myofibers will cease to fire and stop contracting once Cl^{-} efflux hyperpolarization of the myocardium reaches approximately +40 mV. Since 90% of the transplanted myoblasts developed to become cardiomyocytes or heterokaryotic cardiomyocytes and only a small amount of skeletal myofibers were formed, the electrophysiological treatise explains why none of the subjects in the current study experienced malignant arrhythmia from the myoblast transplantation (Law et al. 2004a, b, 2006).

Abraham et al. demonstrated in vitro that connexin 43 was expressed when exogenous connexin 43 gene was transferred into myoblasts (Abraham et al. 2005). Electrophysiological studies had also found that there was synchronized instantaneous calcium current between skeletal muscle myotubes and adjacent cardiomyocytes, further confirming that after transplantation, cardiomyocytes, heterokaryotic cardiomyocytes, and skeletal myotubes were simultaneously activated through the same excitation-contraction coupling. Simultaneous contraction could not only promote the formation of gap junctions, but also effectively reduced the incidence of arrhythmia. In addition, a sodium ionic current was detected (Zebedin et al. 2007) confirming that some myoblasts developed to become myotubes and immature myofiber (Law et al. 2004a, b, 2006). Skeletal myofibers are known to adapt to the frequency of electric excitation to which they are subjected. Under the influence of hormones and slow contractile activity of

the heart, these immature skeletal myofibers developed characteristics of cardiomyocytes.

The regenerative heart with myoblasts was endowed with a greater number of myogenic cells capable of mitosis and was prepared to regenerate upon injury. These cells produced more contractile filaments to augment heart contractility. The latter is fundamental to the quality of life and the life span of patients suffering various forms of cardiovascular diseases. Being pluripotent, embryonic or adult stem cells exhibit uncontrolled differentiation into various lineages to produce bone, cartilage, fat, connective tissue, skeletal and heart muscles. Until scientists can accurately define the specific transcriptional factors and pathways to guide stem cell differentiation into adequate quantity of cardiomyocytes, the use of stem cell injection into the human heart would have a risk-benefit ratio much higher than the use of myoblasts.

Myoblasts are differentiated cells destined to become muscles. Further studies are necessary to better define the efficacy of myoblast transplantation itself, preferably through transplanting male donor myoblasts into female subjects, and using the Y chromosome to track the development of implanted allogeneic myoblasts in the host. Position papers in 2017 highlighted numerous developing cell therapies for severe heart failure with neither governmental approval nor endorsement (Fernandez-Aviles et al. 2017; Mathur et al. 2017). Heart transplant has remained the generally accepted treatment for end-stage patients. With an estimate of over 50 million heart failure patients worldwide, only a few thousand donor hearts were available for transplants last year. Patients who survive heart transplants need to be immunosuppressed for life with not only compromised quality of life but also constant life-threat of COVID-19 (Law 2020a, b, c).

Perspectives

The 35% to 45% relative increases in LVEF at 1 year after MTT reported independently by several teams (Hagege et al. 2003; Dib et al. 2009; Veltman et al. 2008; Law 2016; Smits et al. 2003; Ince et al. 2004; Law et al. 2003; Rosinberg et al. 2005) are highly significant. This has never been achieved with any pharmaceutic or therapeutic modality in the treatment of ischemic cardiomyopathy and heart failure. Such significant increases in LVEF would most likely improve the quality of life and extend the life span of the patients. MTT is the most promising treatments for heart diseases in the horizon (Dib et al. 2009; Veltman et al. 2008; Law 2016).

AMT was used as a last resort for these end-stage subjects. The key methodologic limitation to prevent AMT for widespread usage is the myoblast manufacture of $>$90% purity to minimize immunologic reactions. Our institute has long overcome this limitation. In the Western world, myoblast autograft is a norm, largely because it escapes immunosuppression, a step that the FDA and EMA have favored. However, autografts often provide genetically abnormal cells, especially for diabetic or dystrophic cardiomyopathic patients. Cyclosporine immunosuppression for 3 weeks a minor inconvenience for the HF patients when life-prolongation can be extended for up to 2 years using allografts or AMT as in the current study.

AMT is much less invasive than a heart transplant. The regenerative heart with myoblast allograft is the patient's very own and requires only 3 weeks of immunosuppression. At a small fraction of the cost of a heart transplant, the regenerative heart with allogeneic myoblasts promises lower healthcare spending if proven safe and efficacious.

Conclusion

AMT in adjunct use with CABG and cyclosporine demonstrated that cell survived and engrafted in patients with ischemic cardiomyopathy; in this small study, the cell transplant was safe. It was demonstrated to have sustained the life spans to 2 years postoperatively on ten end-stage HF patients having 2.21-mm mean thinnest wall thickness and ventricular aneurysms, with significant improvement in LVEF, NYHA cardiac function, viability, and quality of life. The improvement in heart function and quality of life could be secondary to combined effect of CABG and AMT. A larger randomized clinical trial is required to confirm the efficacy.

Angiomyogenesis

Animal experimental data had culminated that myoblasts transfected with vascular endothelial growth factor 165 ($VEGF_{165}$-myoblasts), when injected intramyocardially, were potential therapeutic transgene vehicles for concomitant angiogenesis and myogenesis to treat heart failure and ischemic cardiomyopathy (Ferrara 2001; Haider et al. 2004c; Ye et al. 2004, 2005a, b, 2007, 2008). $VEGF_{165}$-myoblasts are second-generation products of genetic cell therapy of MTT capable of replenishing live cells and genetically repairing degenerative myofibers.

Following transplantation, they survived, developed, and functioned to revitalize degenerative myocardium in heart failure and ischemic cardiomyopathy animal studies. The safety and efficacy of $VEGF_{165}$-myoblasts transduced using adenoviral vectors, nanoparticles, or liposomes were compared. We envision that $VEGF_{165}$-myoblasts will provide better outcome than their non-transfected counterparts.

Therapeutic angiomyogenesis has potential application to a host of fatal and debilitating diseases and conditions. A nonviral vector gene delivery approach provides a safer alternative to overcome the untoward effects of viral vectors (Ye et al. 2007, 2008). CD liposome transfected about 7.99% of primary myoblasts under optimized transfection condition. Though the gene transfection efficiency was only 7.99%, the $VEGF_{165}$ transgene expression efficiency was sufficient for therapeutic angiogenic gene delivery for the injured heart. The study highlights the feasibility, safety, and efficacy of CD liposome-mediated $VEGF_{165}$ transfection with myoblasts for angiomyogenesis in cardiac repair.

Reduced myocardium apoptosis, improved wall thickness, increased neovascularization and regional blood flow of the infarcted myocardium together resulted in improved heart function. The CD liposome-based gene delivery approach may have clinical relevance and open a new concept for nonviral angiogenic gene delivery for the treatment of ischemic heart disease (Ye et al. 2008).

Autonomous Robotic Cell Injection Catheter System

Cell transfer therapy has undergone significant progress and has been studied extensively in clinical trials. These types of transferred cells consist of embryonic stem cells, induced pluripotent stem cells, human umbilical cord cells, fetal cardiomyocytes, skeletal myoblasts, resident cardiac stem cells, bone marrow-derived stem cells, and mesenchymal stem cells.

The operation of existing catheters used for cell delivery into the infarct boundary zones of the left ventricle is far from optimal. Injection catheters available are handheld devices operated manually through an inner needle and a distal electrode having tip deflection and torque capabilities. Despite a hefty learning curve, interventionists often encounter difficulties in catheter stabilization and infarct detection, resulting in lengthy operation times and imprecise injections. This myogenic cell injection catheter and method was originally patented in 2005 by Law PK. A design incorporating robotic positional control, feedback signals/images, and an adaptable algorithmic sequence for automation to overcome these problems was examined. The design provides the basis for the construction of a remote cell injection catheter with moments of autonomy to assist the physician to deliver more efficient cell transfer catheterizations (Cheng and Law 2017a, b).

Remote and robotically actuated catheters are the stepping stone toward autonomous catheters, where complex intravascular procedures can be performed with minimal interference from the physician. A concept for the positional, feedforward control of a robotically actuated intramyocardial cell injection catheter was proposed and tested. The prototype for the catheter system was built upon a needle-based catheter with a single degree of deflection, a three-dimensional printed handle combined with stepper motors, and the Arduino microcontroller platform.

A bench setup was used to mimic a left ventricle operation starting from the femoral artery for the injection of committed myogenic cells or undifferentiated stem cells into a myocardial infarct boundary. Using Matlab and an open source video modeling tool, Tracker, the planar coordinates (y, z) of the catheter position were analyzed and a feedforward control system was developed based on empirical models. Using the Student's t-test with a sample size of 26, it was determined that for both the y- and z-axis, the mean discrepancy between the calibrated and theoretical coordinate values had no significant difference compared to the hypothetical value of $\mu = 0$.

A feedforward empirical model for the planar positioning of a robotically controlled cell injection catheter has been established. The calibrated coordinate control was not significantly different from the theoretical coordinates, while the unmodified values show strong significant difference. This proof-of-concept investigation leads to the possibility of further developing a feedforward control system in vivo using catheters with omnidirectional deflection. Feedforward positional control allows for more flexibility in the design of an automated catheter system where problems such as systemic time delay may be a hindrance in instances requiring an immediate reaction (Cheng and Law 2017a, b). Whereas this design appeals to catheter cell injection in the left ventricle, it is plausible to apply similar techniques or robotic

functions to other cardiovascular catheter procedures. Components in this design, such as the stabilizing OCG, robotic IOC, or the robotic arm manipulating the IOC, can all be developed independently and used to assist with other procedures. Combining these designs to form a novel procedural system resolves several of the issues involving direct intramyocardial cell injections. As cardiovascular disease is the leading ailment in the world and open heart surgery is a morbid solution for the direct injection of stem or myoblast cells, it is essential to establish an optimal noninvasive solution for this therapy.

Future work includes the development of an omnidirectional catheter with force sensing capabilities. A feedforward model for the positioning of the omnidirectional catheter should be derived using kinematic or empirical models. Following bench experiments, porcine trials should be used to determine the accuracy and precision of the robotic system in a variable and moving environment. Once feedforward control is established in vivo, feedback control should also be implemented to optimize the system. Outside of automated positional control, other future experiments include the development of automated control for the needle and injection process, the determination of infarct boundary zones via recording electrodes, and determining contact force thresholds whenever the catheter tip encounters an intracardiac structure. Combining these functions into a step-by-step sequence, a complete or partially autonomous catheter injection procedure can be developed where minimal intervention from the physician is required.

Cellular and Molecular Phases of MTT Development

All of the MTT works described above relied on increased muscle strength, more myofibers, and better cell structure and protein replenishment as monitoring endpoints. The beneficial effects were mediated through donor cell survival, development, and functioning per se. Gene transcription and translation leading towards genetic repair had not been seriously addressed in the clinical prophylaxis and treatment of multiple gene defects such as in dystrophic or diabetic heart diseases. Although there had been awareness and understanding that disease symptoms of a variety of muscle diseases could be alleviated with MTT, such achievement by changes in the quality and quantity of direct transcripts of multiple genes had not been demonstrated.

MTT Corrects Gene Defects in Type II Diabetes

With cyclosporine, human myoblasts survived extensively in diabetic mouse skeletal muscles at 12 weeks after MTT (Ye et al. 2009). Glucose tolerance test showed a significant decrease of blood glucose in the mice of KK myoblast group compared to the KK control (Ma et al. 2013). Transcriptional patterns of insulin signaling pathway showed alterations in KK myoblast as compared with KK control group (23 genes). Transcriptional patterns of mitochondrial biogenesis and function also

had alterations in KK myoblast as compared with KK control group (27 genes) (Ma et al. 2013). Using array analysis, it was shown for the first time that changes in the quantity and the quality of direct transcripts of *multiple* genes could result in disease relief using MTT on a genetically abnormal animal model of Type-II diabetes. These changes not only identified the multiple gene defects in Type-II diabetes but could also be used as tools for identification and selection of prophylactic or therapeutic agents or lead drug compounds (Ma et al. 2013). For the first time, at least in the mouse, Type II diabetes was shown to be a polygenic disease involving at least 50 gene defects.

Type II Diabetes

Diabetes is a leading cause of kidney failure and nontraumatic lower-limb amputations among adults in the world. In 2010, the USA was estimated to have spent $198 billion on diabetes treatment (Zhang et al. 2010). An estimated 285 million adults had Type II diabetes making up about 90% of diabetes cases in 2010 (Vijan 2010). Diabetes affects ~25% of Western populations, steadily increases (Mokdad et al. 2001), and is an important cardiovascular disease risk factor (Haffner et al. 1998). Epidemiological and twin studies have clearly indicated a major polygenetic factor in the development of insulin resistance, a key feature of Type II diabetes, which was influenced also by environmental factors (Guillausseau et al. 1997; Stern 1999).

Type II diabetes, also called non-insulin-dependent diabetes mellitus, can be traced to the genetic defects of the glucose transporter 4 (GLUT4) and the insulin-regulated aminopeptidase (IRAP) genomes (Cushman and Wardzala 1980; James et al. 1988, 1989; Stenbit et al. 1997; Morgan et al. 2011). Such a genetic defect is manifested in reduced GLUT4 storage vesicle (GSV) exocytosis and endocytosis trafficking, resulting in a significant reduction in uptake of blood glucose into muscle fibers and adipose tissue, where 75% of the body's glucose metabolism normally occurs. In Type II diabetes patients, normal or even elevated levels of plasma insulin would not elicit normal glucose uptake and high blood sugar persists.

MTT in Type II Diabetes Human Study

A pioneering feasibility/safety study of administering 25-billion myoblast allografts into the skeletal muscles of Type II diabetes patients led the way in developing a genetic treatment for the disease (Law et al. 2004c). The procedure was shown to be safe for both subjects.

A potential genetic treatment of the disease would involve MTT similar to the 50-billion myoblast protocol used to treat muscular dystrophy. It would consist of culturing genetically normal, immature muscle cells called myoblasts, derived originally from a 2 g skeletal muscle biopsy from a healthy, young, male donor free of blood-borne pathogens, and injecting these allogeneic myoblasts with host

serum at approximately 10^8 cells/ml into 80 major muscles of a diabetic patient. Cyclosporine would be used for 2–3 weeks as an immunosuppressant.

MTT Use in Drug Discovery

A drug discovery assay would comprise detection of increased gene transcription of one or more of a group of 22 genes involved in insulin signaling pathway after MTT (Ma et al. 2013). Assays of RNA transcripts demonstrating the qualitative or quantity change of RNA transcription would be used to select a lead drug compound for clinical promise or use in disease (such as diabetes) prophylaxis or alleviation of symptoms. In addition, bioassay of a gene transcription product activity would be used to select the lead drug compound. The gene would be one or more selected from the group consisting of Acaca, Aebp1, Cfd, Gpd-1, Jun, PPAR gamma, Ptpn1, and UCP1 as identified (Ma et al. 2013).

Likewise, another drug discovery assay might comprise detection of increased gene transcription of one or more of a group of 27 genes involved in mitochondrial biogenesis and function. Assays of RNA transcripts demonstrating qualitative and/or quantity change of RNA transcription would be used to select a lead drug compound for clinical promise or use in disease (such as diabetes) prophylaxis or alleviation of symptoms. Bioassay of a gene transcription product activity would be used to select the lead drug compound. The gene would be one or more selected from the group consisting of Bcl211, Cox10, Cpt1b, Slc25a22, Slc25a25, Stard3, Timm17b, and Tomm40 (Ma et al. 2013).

Multiple Usage of MTT in Genetic Diseases

A variety of uses of this technology were described, including that for disease treatment, disease prevention, drug discovery, and selection of superior cells and clones for therapy. MTT resulted in gene transcript changes in multiple pathways. Linking the MTT technology development from DMD, cardiomyopathy, and Type II diabetes, MTT demonstrably mediated its effect through transfer of the normal myoblast nuclei that supplied the complete human genome, in addition to just replenishing the normal counterpart(s) of the missing gene(s) or the aberrant gene (s). The replacement genes then transcribed to produce the necessary proteins or factors for genetic repair.

Preliminary Safety and Efficacy Data of MTT in Cancer Treatment

In 2017, Law et al. reported the preliminary safety and efficacy of MTT in human cancer treatment. Evolution of placental mammals over the past 160 million years witnessed the relative sparing of muscles from cancer attacks (Law et al. 2017). In nude mice with human gastrointestinal or lung tumors, and human subjects with liver, lung, or gastrointestinal tumors, intra-tumor implantation of allogeneic human

myoblasts induced cancer apoptosis, inhibiting metastasis and tumor growth. We postulated four mechanisms of cancer apoptosis: (a) myoblasts releasing tumor necrosis factor-α (TNF-α); (b) nutrient deprivation; (c) local inflammatory and immunologic attacks; and (d) prevention from metastasis. These basic and clinical studies demonstrated preliminary safety and efficacy of intra-tumor myoblast implantation in the development of prevention and treatment for cancer, now the number one disease killer of mankind.

Direct injection of allogeneic human myoblasts at 100 million per milliliter of host serum into the solid tumor without immunosuppressant was preferred, although myoblast concentration might vary from 75 to 250 million per milliliter. Exposing the allogeneic myoblasts to 100% host serum primed the myoblasts for proliferation. Implantation of this mixture into the tumor constituted serum restriction, a condition that terminated mitosis and induced cell fusion to occur.

Identification of the Polygenic Defects of Various Cancer

Development of cancer treatment in the last three decades witnessed approaches not dissimilar to the blind men reporting on the elephant. Molecular geneticists proceeded on specific and often single transcriptional pathways without identifying and correcting the complete anomaly. Whereas cancer of various types must have involved multiple gene defects by themselves, it would be of scientific logic to extend the studies of MTT, array analyses, and gene transcriptional pattern changes described for Type II diabetes to cancer. These studies are on their way. Meanwhile, additional data is reported for understanding the basic mechanisms of cancer prevention and treatment using MTT.

Myoblasts Inhibit Metastasis, Tumor Growth, and Induce Cancer Apoptosis

In a series of "competition to survive" experiment, human myoblasts and Fadu cancer cells (Fadu) were plated at 20 to 1 ratio in cell number and co-cultured with super medium. Cells were fed with new medium on day 3 and day 7 only during which both cell types underwent mitosis and proliferated to confluency. Figure 6 depicts myoblasts inhibited Fadu cells from metastasis on day 12, and induced cancer cell apoptosis beginning at day 18 and completely by day 24, presumably by secreting TNF-alpha, and by outcompeting Fadu for nutrients. Day 24 Fadu control culture without myoblasts showed substantial Fadu cell survival despite no feeding since day 7, indicating myoblasts inhibited metastasis and induced cancer apoptosis in the co-cultures.

To confirm the above contention, the super medium was substituted with fusion medium on day 7, thus hastening fusion of myoblasts in addition to further subjecting the cells to deprivation of nutrients. The switching from super medium to fusion medium constituted a condition of serum deprivation because the fusion medium contained only one-fifth of the serum concentration as in the culture medium. Serum deprivation terminated the mitotic cycle of the myoblasts, and

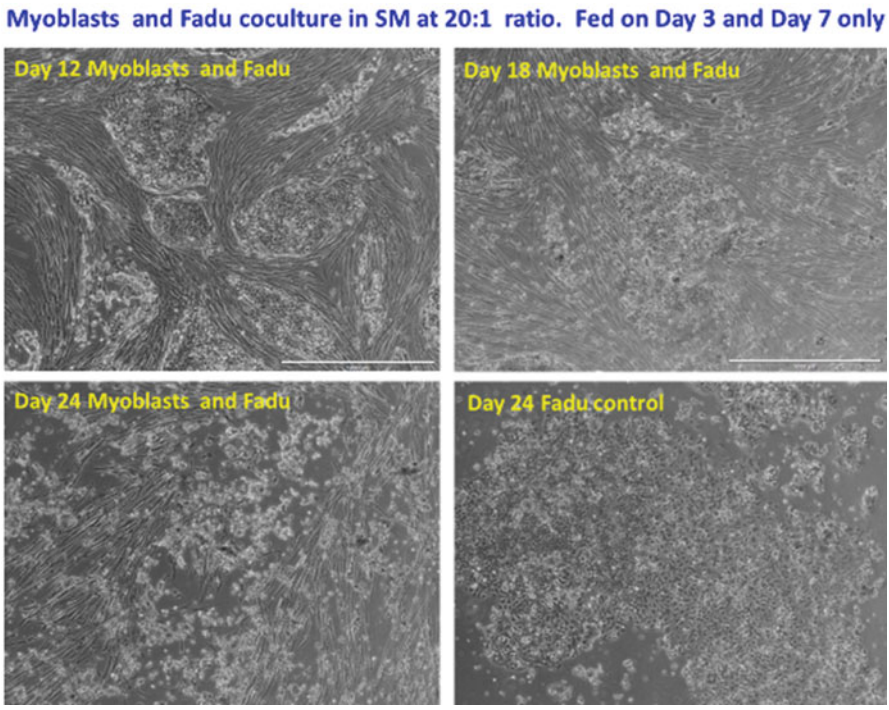

Fig. 6 Human myoblasts and myotubes (spindle- shaped) surrounded human Fadu cancer cells (round) in co-culture, preventing them from metastasis on day 12, and killing them beginning at day 18 and completely by day 24. Dark cells were alive, whereas white cells were dead. Bar scale = 100 micro-meters

initiated the developmental process of natural cell fusion towards myotube formation. It is during the early phase of myoblast fusion that the cell membrane breaks, releasing 25 times of the basal level of tumor necrosis factor-α (TNF-α) to induce cancer apoptosis. Using immunocytochemistry, DAPI was used to label all cell nuclei, cleaved-caspase 3 to label apoptosis, and desmin to label myoblasts and myotubes (Fig. 7). Apoptosis analyses on day 24 were conducted using Annexin V/PI (Fig. 8). Results indicated that more cells underwent apoptosis when myoblasts were co-cultured with Fadu cells than when Fadu controls, regardless whether in the super medium or in the fusion medium.

Myoblasts Fuse with Cancer Stem Cell

Given the intra-tumor confluent milieu where dividing myoblasts and cancer stem cells were plentiful, natural and /or controlled cell fusion must have occurred. Genetic deficiencies of the cancer stem cells were now remedied or complemented by the presence of gene transcripts of the normal myoblast genome supplied through MTT. This genetic mosaicism had previously been demonstrated.

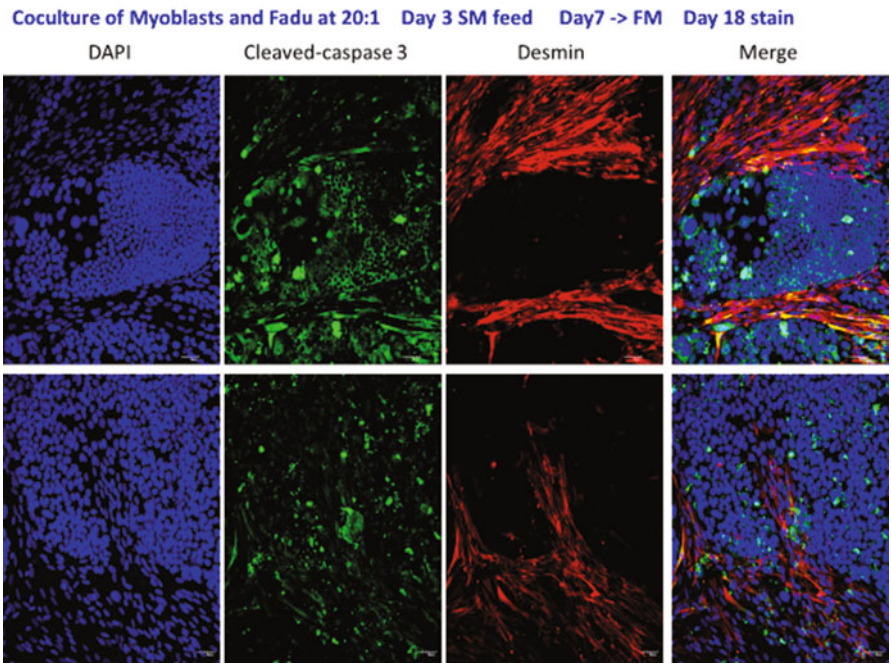

Fig. 7 Human myoblasts and myotubes (red) surrounded human Fadu cancer cells (blue) in co-culture, preventing them from metastasis, and killing them (green) at day 18. Bar scale = 20 micro-meters

Parlakian et al. reported that myogenic cells exerted pronounced effects upon co-culture with metastatic melanoma (B16-F10) or carcinoma (LLC1) cells including conversion to the myogenic lineage in vitro and in vivo, as well as inhibition of melanin production in melanoma cells coupled with cytotoxic and cytostatic effects (Parlakian et al. 2010). Tumor suppression assays revealed that the muscle-mediated tumor suppressor effects did not generate resistant clones but functioned through the down-regulation of the transcription factor MiTF, a master regulator of melanocyte development and a melanoma oncogene. These results pointed to skeletal muscle as a source of therapeutic agents in the treatment of metastatic cancers. The cell fusion process constituted one of the five mechanisms to support MTT efficacy in treating cancer (Law et al. 2017).

Plausible Sequence of Events

Myoblasts' unique characteristic shared only with cardiac and smooth muscle cells was natural cell fusion, through which myoblasts, at the end of their mitotic cycle, underwent cell membrane breakage, releasing large but natural quantity of cancer-killing tumor necrosis factor-a (TNF-α) and possibly other TNFs into the micro-environment. Associated only with myoblasts and no other bodily cells, TNF-α

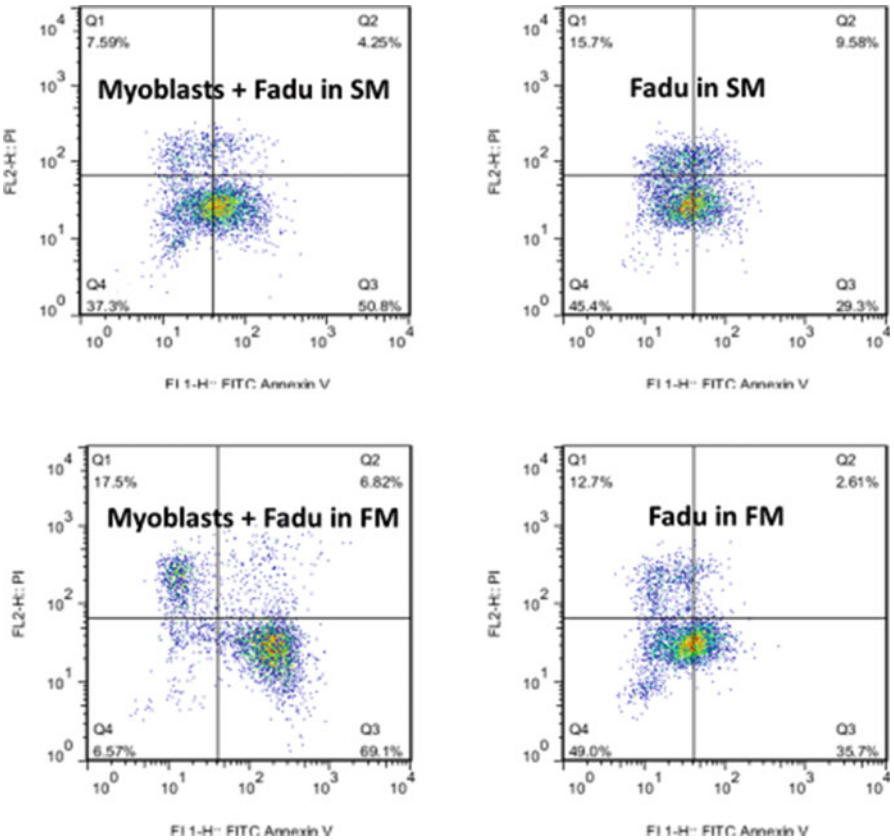

Fig. 8 Annexin V/PI recordings at day 24 to demonstrated that more cells underwent apoptosis and cell death when Fadu cells were co-cultured with myoblasts than control itself, regardless whether in the super medium (SM) or in the fusion medium (FM). Apoptosis and cell death were more in the fusion medium with fewer nutrients

was formerly called cachectin, a promotional factor in myoblast development into myotubes. The second phase of cell fusion was accomplished by massive sarcolemma formation, enclosing 200–500 myoblast nuclei into one myotube. Competition for nutrients and oxygen against cancer cells within the tightly encapsulated tumor was fierce, resulting in death of cancer and myogenic cells. Each of the myotube had to be vascularized and innervated to survive, failing which the myotubes would disappear, leaving vacuoles and empty spaces within shrunken tumors as compared to control (Law et al. 2017). Furthermore, allogeneic myoblast implantation triggered inflammation and local immune response, killing myoblasts and cancer cells indiscriminately. Cancer cells also became non-metastatic as being "wrapped" with myoblasts (Fig. 6). Stolting et al. also reported that myoblasts inhibited prostate cancer growth by paracrine secretion of TNF-α (Stölting et al. 2013).

MTT in Breast and Uterine Cancers

Breast cancer is perhaps the most preferent malignancy in which remedy is resorted to surgery. The trauma was physical and emotional, and metastasis often ensued. MTT bypasses the trauma without leading to metastasis, and when proven safe and efficacious, will be a good candidate for treatment for breast cancer, and uterine cancer likewise. In addition to inhibiting metastasis, tumor growth and inducing cancer apoptosis, myoblasts can augment the size and shape of the breast and strengthen the wall of the uterus.

In investigating MTT safety and efficacy in treating endometrial carcinoma, neoplasm was produced in 30 nude mice that were divided into three equal groups, then given intra-tumoral injections of myoblasts (1×10^7 cells in 0.2 ml), saline (0.2 ml), and intraperitoneal injection of Cis-platinum (3 mg/kg body weight), respectively. Neoplasm inhibition rate (NIR) of the MTT group and the chemotherapy group were 85.95%, 64.33%, respectively, in 4 weeks. The positive rate of PTEN in the control, chemotherapy, and MTT groups were 30%, 40%, 60%, respectively.

Tumor volume (mm^3)

	Before	After treatment	NIR
Control	38.27 ± 2.58	154.02 ± 13.66	–
Chemotherapy	35.99 ± 1.32	54.94 ± 10.84	64.33%
MTT	37.05 ± 2.93	21.64 ± 7.56	85.95%
Chi-square test before treatment($F_1 = 1.85$, $P_1 = 0.18$ $P>0.05$)			
Chi-square test after treatment($F_2 = 315.11$, $P_2<0.01$)			

Body weight of nude mice (g, $\bar{x} \pm SE\ \bar{x}$)

	Before	After
Control	17.16 ± 0.73	16.23 ± 0.31
Chemotherapy	17.02 ± 0.40	17.26 ± 0.50
Experiment	17.05 ± 0.38	19.50 ± 0.49
Chi-square test before treatment ($F = 0.16$, $P = 0.853$)		
Chi-square test after treatment ($F = 116.31$, $P<0.01$)		

Results of the study indicated that myoblasts was more effective than Cis-platinum in inhibiting the proliferation of endometrial carcinoma in nude mice (Fig. 9).

Conclusion

Since the seminal work of Law in 1995 disclosing the results of co-cultures of normal human myoblasts and malignant melanoma (CRL6322) cells (Law, Myoblast therapy for mammalian diseases), significant amount of research has been conducted on MTT in cancer treatment. These basic (Law et al. 2017; Parlakian et al. 2010; Stölting et al. 2013; Law, Myoblast therapy for mammalian diseases) and clinical studies (Law et al. 2017) demonstrated the preliminary safety and efficacy of intra-tumor myoblast implantation in the development of prevention and treatment

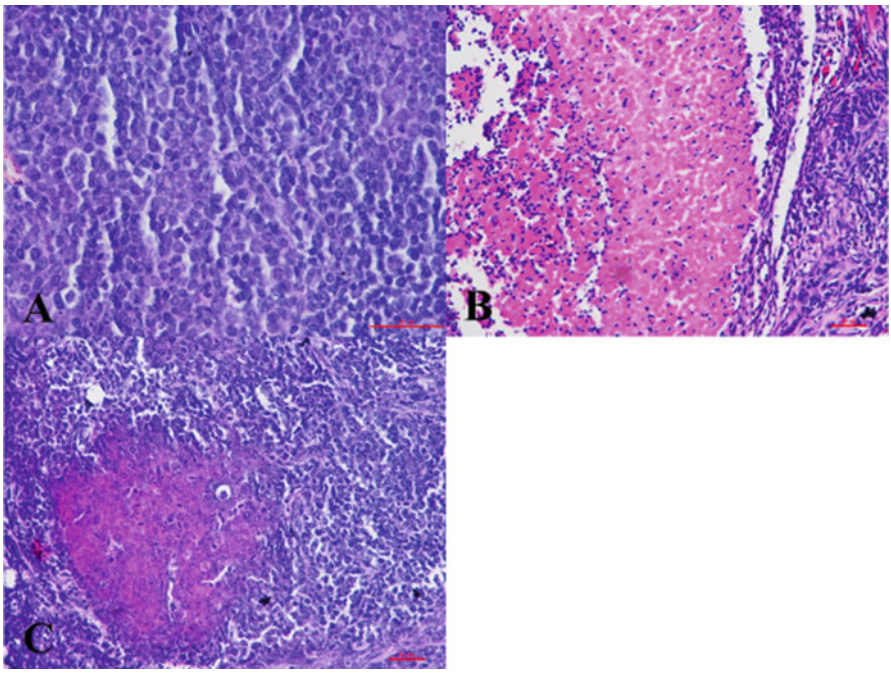

Fig. 9 Neoplasm of (**a**) control, typical neoplasm cells, and atypia cells were observed. (**b**) Myoblast treated, cellular pyknosis, karyorrhexis, and apoptotic necrosis were observed and (**c**) Cis-platinum treated, little of necrosis tissue was observed. H&E. Microscopic magnification x400

for cancer. In face of massive dying patients, the benefit/risk ratio will favor well-designed clinical trials to be conducted at their earliest, including randomized, double-blind studies.

Antiaging Aesthetica

It is in this arena that MTT finds wide application. Beauty is a physical attribute that often enhances one's self-confidence, career, and quality of life. The physical parameters of appearance are size, shape, tone, color, luster, texture, consistency, and density. These parameters deteriorate in every organ according to the genetically programmed degeneration of aging. Skin, skeletal muscles, facial structures, bones, teeth, and breasts are the organs that directly affect external appearance, whereas weakening of anal and urinary sphincter muscles can even affect excretory function. The patented MTT is at the forefront of regenerative medicine using live cells to enhance the parameters of appearance (Law, Myoblast therapy for cosmetic treatment; Law, Biologic skin repair).

In Law's Enchanting World of MTT

Human average life span will be 100 with MTT. People will be healthy, youthful with high quality of life. To reach there, people, especially people in charge, need to be educated with biologics and regenerative medicine. Many are still toiling with chemicals and molecular biochemistry.

Personalized Medicine for Each Family

A myoblast cell line of 100 billion or more will be established from 2 g of muscle biopsy from the healthiest male child of each family, to be used in prevention, treatment, or beautification of every family member.

Biologic Skin

The polygonal skin fibroblasts are about 15 times the size of the myoblasts and produce a rough body cover. Cancers are common in skin but rare in muscle. Myoblasts, because of their small size, spindle shape, and resilience, can grow within wrinkles and on skin surfaces, thus enhancing the color, luster, and texture of the skin "plated" with them (Law, Biologic skin repair). Biologic creams are formulated to promote cell survival, growth, and development to enhance the color, luster, density, and texture of the skin. Thus, layers of biological skin consisting of pure myoblasts and myotubes can eliminate skin defects and blemishes, especially after laser removal.

Body Sculpture

Intramuscular injection of myoblasts can augment the size, shape (Law, Myoblast therapy for cosmetic treatment), consistency, tone, and strength of muscle groups, improving the lines, contours, and vitality from the sculpture for a youthful appearance. Myoblast technology can be used for cosmetic enhancement such as bodybuilding and in tissue implants for breast, buttock, or facial augmentation.

The myoblasts can also be injected subcutaneously as live cell filler. Unlike the noncellular collagen, which is absorbed in 3–6 months after injection, injected myoblasts are cells that will survive and last for tens of years within the host. Myoblasts are endogenous to the human body and have been proven safe in clinical trials involving over 280 muscular dystrophy patients and 300 heart failure cases worldwide. Myoblasts will not cause cancer like silicone or burst and absorbed like saline or collagen implants.

Be More Macho

The distribution of oxygen and nutrients to the peripheral organs is significantly reduced for people aged over 45. In developing treatment for human myocardial infarction, we have grown five times more blood capillaries and muscle simultaneously using human myoblasts transduced with angiogenic factors (Ye et al. 2004, 2005a, b; Ferrara 2001; Haider et al. 2004c). In addition to their application to treat heart diseases, these cells can potentially be used to treat male/female impotency and baldness and to produce redder, thicker lips and pinker face because of the higher density of capillaries within layers of myogenic cells after myoblast treatment. The latter serves as a fertile ground to seed new hair follicle cells on the bald head or other body parts to give the desirable hair style, color, density, and consistency.

Be More Feminine

Stressed urinary incontinence (SUI) and fecal incontinence (FI) are embarrassing dysfunctions that inhibit socializing of innumerous numbers of women aged 45 upward. Birth of children has left these ladies, some holding important offices, with weakened internal and external sphincter muscles. Publication over 1100 cases had demonstrated that MTT is safe and efficacious for SUI (Law, Biologic skin repair) and FI. In conjunction with treating SUI, more myoblasts can be implanted into the vaginal wall to enhance tightness and contractile force during sexual intercourse. Current surgical and cauterizing techniques often left scars that created discomfort for both partners. Myoblast implantation over the pubic bone provides not only a sexier appearance, but also a "shock absorber" on impact, thus reducing the piercing discomfort caused by the skinny yet attractive ladies. And for that special partner and moment, a hymen can be reconstructed from layers of myotubes cultured and patched on with biologic glue. Similar restructuring of the larynx can produce a sexier voice of higher pitch, a tympanum for the traumatized eardrum, a cellular patch for esophageal and/or stomach ulcers.

Tendon Repair for Injured Athletes

Myoblasts constitute a better biomaterial than tenocytes in synthesizing stronger and smoother tendons for transplantation repair. These tendons exhibited greater stiffness, tensile strength, Young's modulus, and could handle higher maximum load (Chen et al. 2012). One can envision MTT developing into sport and military medicine in muscle, tendon, bone, cartilage, and joint repairs.

Loose Teeth and Bone Fracture Repair

It has long been known that osteoblasts culture is nonproductive. Bone morphogenetic protein-2 converted myoblast differentiation pathway into osteoblast lineage (Katagiri et al. 1994), thus providing a good source for osteoblasts useful in

anchoring loose teeth. In combination with the second-generation product $VEGF_{165}$ myoblasts, they are being assessed for treating bone fractures and firming up loose teeth.

Stimulation Therapies and Fractal Dynamics to Complement MTT Treatment of Diseases

Therapeutic stimulation methodologies based on inherently fractal nature of physiologic dynamics involve the use of electrical currents, electromagnetic fields, temperature change, ultrasound, light, and so forth. These stimulation therapies can be categorized into three main modalities: electrical stimulation modalities, thermal modalities, and nonthermal modalities. Electrical stimulation modalities include therapeutic techniques where electrical current is directly applied to the body of treated subject. Direct application of electrical current to the brain also falls under this category. Thermal modalities consist of stimulations that induce temperature change on the body for therapeutic effects without the direct transfer of electrical current. Nonthermal modalities functions through energy transfer without directly applying electrical current and without the effects of temperature change. A fourth miscellaneous category for stimulation techniques consists of the stimulation effects of music along with physical stimulation as in massage therapy. Common to most of these therapeutic strategies is that the stimulation is delivered at certain fixed periods or frequencies. We introduce some rudiments of fractal dynamics, and the notions of self-similarity, scale-invariance, and long-range correlation or memory in the dynamics of a system (Cheng et al. 2014). Our group present evidences that fractal dynamics is commonly observed in healthy physiological systems while unhealthy systems are shown to veer away from fractal dynamics towards periodic or random motion. This difference in dynamics can be observed in many biological signals such as in neural activity, heart rate variations, and breathing patterns. It is being proposed propose that an optimal stimulation technique should thus be one that encourages an unhealthy, non-fractal pathological system towards a healthy, fractal dynamic. Given the ubiquity of fractality in healthy biological dynamics, it is being argued that a fractal pattern of stimulation is a more optimal approach to functional restoration than the widely used conventional periodic stimulation, which may further consolidate the existing pathological dynamics (Cheng et al. 2014).

MTT Replenishes Mitochondria and Regenerates Energy Network of Life

In hereditary conditions of aging (Campisi et al. 2019), DMD and various fatal genetic diseases, one transits from being normal to sick unto death. The feelings of being weak and cold indicate degradation of the energy production network of mitochondria (Law et al. 1983) densely populating the neuromuscular systems that are doing the real heavy lifting (Figs. 10 and 11).

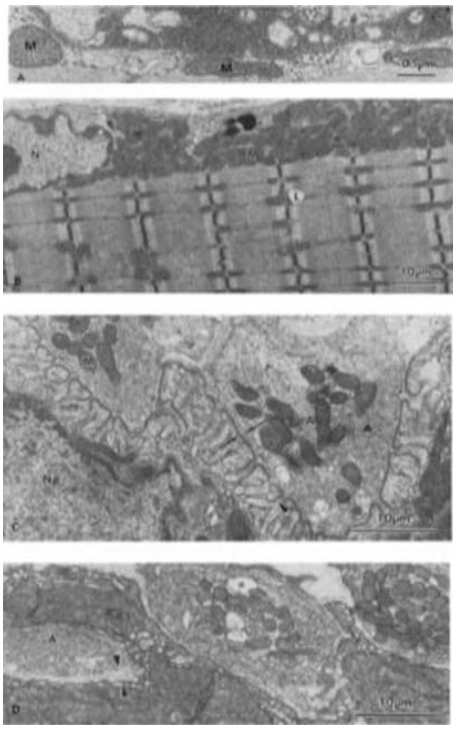

Fig. 10 Electron micrograph of dystrophic mouse myofiber showing (**a**) normal appearing mitochondria-M with cisternae regularly spaced; (**b**) for every nucleus, there were about 100 mitochondria around; (**c**) normal presynaptic terminal or axon-A contained numerous synaptic vesicles and mitochondria-M. The primary synaptic cleft (between arrows) was uniform and was approximately 400 A wide. Numerous secondary synaptic folds were present. The postsynaptic membrane showed thickening (arrow points to right corner). (**d**) Degenerative end-plate with abnormal mitochondria (see Fig. 11), widened primary synaptic cleft (between arrowheads), and the secondary synaptic folds were absent. Thickening of the postsynaptic membrane could not be discerned. (Reproduced with permission from Law et al. Experimental Neurology; 80:361, 1983)

Striated muscles, cardiac muscles, and smooth muscles that occupy approximately 55% by volume of the human body are constantly at work to maintain body temperature and normal metabolism. Degradation of the power network of adeno-triphosphate (ATP) production leads directly to cell apoptosis, malfunction, and death (Law et al. 1983).

With the 50-B MTT into 80 large muscle groups of the body, there was a mean 70% increase in normal myofiber number attributed to donor myoblast fusion after transplantation. Considering that each myofiber contains 200–500 myonuclei, literally trillions of mitochondria from young men are implanted with each 50-billion MTT to regenerate the energy network of life. These are not isolated and manipulated mitochondria that have difficulty in integration into the

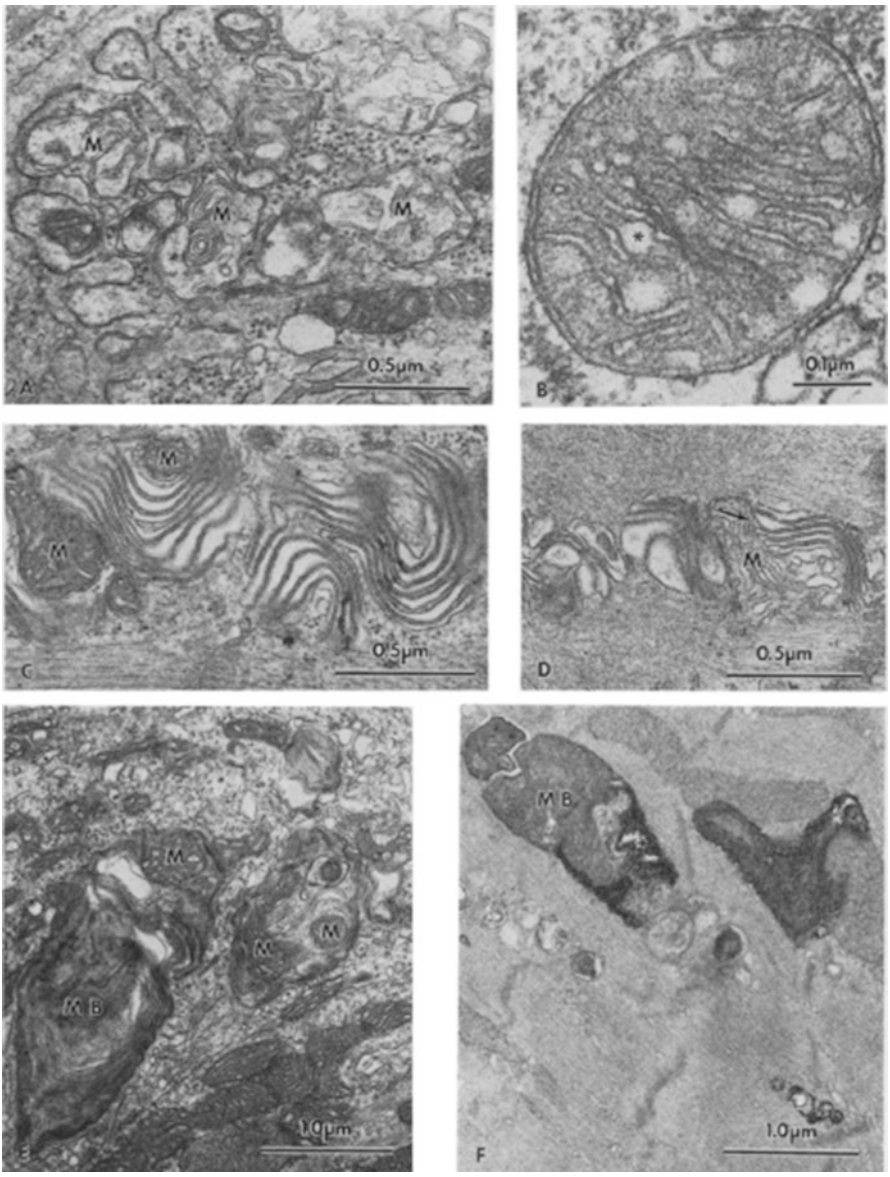

Fig. 11 Abnormal mitochondria (M) and membranous bodies (MB). (**a**) Section of dystrophic muscle fiber containing membranous structures could still be recognized as swollen and disrupted mitochondria. (**b**) Mitochondria with numerous areas of low density (*) in the matrix space. (**c**) Membranous body associated with mitochondria. (**d**) Mitochondrial outer membrane forming a continuum (T) with membranous body. (**e**) Large autophagic vacuole or membranous body containing membranous structures and mitochondria. (**f**) Membranous bodies showing positive acid phosphatase reaction. The asterisk denotes a lead phosphate deposit. (Reproduced with permission from Law et al. Experimental Neurology 80:361, 1983)

human body through mitochondrial transfusion (Sullivan 2021). Try the 50-B MTT on elderly, a DMD boy, or a Type-II diabetic. The protocol was the one that has kept some DMD boys living in their 30s and 40s (1997a, b, c, 1998).

Intellectual Property Portfolio of Professor Peter K. Law

As the pioneering inventor of the MTT platform technology, Professor Peter K. Law personally filed, patented, and currently holds the following inventions governing the compositions, methods, and medical devices associated with MTT applications and research. International collaboration and licensing are available at www.peter@celltherapy.com.

1. Compositions for and methods of treating muscle degeneration and weakness. *WO2018078419A1*; *US5130141A* CN107998150A; EP3315134A1; EP3315134B1; GB2567377A; US2018133260A1; US2019216858A1.
2. Myoblast therapy for mammalian diseases. *WO9618303A1*; *AU5831199A*; *AU748997B2*; *AU4597696A*; *CA2183167A1*; CNZL95192528.8 *CN1146712A*; *CN1127343C*;*CN1477190A*; DE P2116DE01; *EP0743820A1*; *EP0743820A4*; *EP1407788A2*; *FR P2116FR01*; *GB P2116GB01*; IE P2116IE01; *SG74036A1*; *SG99279A1*; *SG99846A1*; *US200203150lA1*; *US6261832B1*; *US7341719B1.*
3. Myoblast transfer therapy for relieving pain and for treating behavioral and perceptive abnormalities. US7166279B2; CA2225185A1; AT241992T; DE69815230T2; EP0898967A1; EP0898967B1; HK1016897A1; IE0898967; JPH11147829A; MX226489; US2002044925A1; US7166279 B2.
4. Automated cell processor. *WO9618303A1 US6261832B1 AU4597696A*; *CA2183167A1*; *CN1127343C*; *CN1146712A*; *CN1477190A*; *EP0743820A1*; *EP0743820A4*; *EP1407788A2*; *SG74036A1*; *SG99279A1*; *SG99846A1*; *US7341719B1.*
5. Instrument for cell culture SG74036.
6. Cardiomyocytes for heart muscles damaged in heart attacks. *WO9618303A1 US2002031501A1*; *AU4597696A*; *CA2183167A1*; *CN1127343C*; *CN1146712A*; *CN1477190A*; *EP0743820A1*; *EP0743820A4*; *EP1407788A2*; *SG74036A1*; *SG99279A1*; *SG99846A1*; *US6261832B1*; *US7341719B1*; US20020031501A1.
7. Myogenic cell transfer catheter and method. *WO0228470A1*; *WO0228470A9 AT345158T*; *AU1123002A*; *CA2422065A1*; *CN1474707A*; *DE60124574T2*; *EP1324802A1*; *EP1324802A4*; *EP1324802B1*; SG95355; *US2005124975A1*; *AU2007202290A1*; *AU2007202290B2 AU2012200651A1*; *AU2012200651B2*; *AU2015202212A1*; *SG95355.*
8. Use of transduced myogenic cells EP19980201068.

9. Cellular transplantation for heart regeneration. *WO03085092A2*; *WO03085092A3*; *WO03085092B1*; *AU2003220562A1*; *AU2003220562A8*; *EP1497410A2*; *EP1497410A4*; *US2003232431A1*; *US200524384A1*.
10. Mechanisms of myoblast transfer in treating heart failure. *WO2004014302A2*; *WO200401430 2A3 AU2003269944A1*; *AU200326994 4A8*; *CA2495112A1*; *CN1688701A*; *CN03824045.9*; *EP1623034A2*; *EP162303 4A4*; *US2006104961A1*.
11. Method and relative composition for controlling cell fusion CN1477190 A/ 2004-02-25.
12. Myoblast therapy for cosmetic treatment. *WO9618303A1 US7341719B1*; *AU4597696A*; *CA2183167A1*; CN03101588.3; *CN1127343C*; *CN1146712A*; *CN1477190A*; *EP0743820A1*; *EP0743820A4*; *EP1407788A2*; *SG74036A1*; *SG99279A1*; *SG99846A1*; *US7341719 B1*.
13. Bioactive implants. *WO200403070 6A2*; *WO200403070 6A3* AU2003272805A1; *AU200327280 5A8*.
14. Methods for producing cardiomyocytes capable of proliferation SG99846.
15. Myoblast treatment of diseased or weakened organs. WO2005020916A2 *US2007009499A1*; *WO200502091 6A3*.
16. Biologic skin repair and enhancement. *WO2004017972A1*; *WO2004017972A8*; AU2003263906A1; *AU2003263906B2*; *CA2496434A1*; *CN100482228C*; *CN1700915A*; *CN03819963.7*; *CN E038199637XS*; *EP1587515A1*; *EP1587515A4*; *SI110581*; *US200605711 9A1*.
17. Disease prevention and alleviation by human myoblast transplantation. US13/ 968,982; *US10449219B2*; *US2015050300A1*; *US2018000867A1 AU2013231029A1*; CN201310455357.4; *CN103550784A*; *CN107648267A*; *CN108042571A*; *EP2837683A1*; *EP2837683B1*; *ES2615553T3*; *HK1248114A1*; *JP2015051969A*.
18. Compositions comprising myoblasts for tumor growth inhibition and prevention of cancer cell metastasis by implantation. *WO2018078419A1*. US2019216858A1; *CN107998150A*; *EP3315134A1*; *EP3315134B1*; *GB2567377A*; *US2018133260A1*.
19. Autonomously controllable pull wire injection catheter, robotic system comprising said catheter and method for operating the same. *WO2018055433A1*; *AU2016423681A1*; *CN107854763A*; *EP3298978A1*; *EP3298978B1*; *GB2567122A*; US10166368B2; *US2018078739A1*.
20. Composition for use as a medicament for the treatment of sudden attacks of COVID-19 and other pathogenic epidemics, sera set, and method for identifying viral antigens and effective antibodies. PCT/IB2020/051964 (pending).

FDA, EMA Approved MTT IND's

FDA currently listed 23 myoblast implantation projects, and EMA listed 6, mostly in Phase II clinical trials (Fig. 12).

FDA	Identifier NO.		Identifier NO.
1	NCT00975234	13	NCT00050765
2	NCT00908622	14	NCT00375817
3	NCT02196467	15	NCT00102128
4	NCT01355133	16	NCT00054678
5	NCT01119820	17	NCT01298375
6	NCT02075216	18	NCT00472069
7	NCT00773227	19	NCT02208713
8	NCT02878694	20	NCT01963455
9	NCT00626314	21	NCT02156934
10	NCT01523522	22	NCT01953315
11	NCT03802279	23	NCT01011777
12	NCT00526253		

EMA	EudraCT Number	Medical condition
1	2005-004034-41	Ischemic Heart Failure
2	2005-003759-11	Fecal (anal) inconinence caused by ruptures/ traumas of the external anal sphincter
3	2006-000679-14	Pacientes con infarto antiguo de miocardio (patients with old myocardial infarct)
4	2013-004140-32	Acute Muscle Injury Type 3A-3B (Munich classification) or type II (Classification of Otto Chan), confirmed by radiological diagnosis and clinical agreement.
5	2007-004695-39	Duchenne Muscular Dystrophy
6	2009-012389-30	Stress urinary incontinence due to intrinsic sphincter deficiency (ISD).

Fig. 12 FDA listed 23 myoblast implantation projects, and EMA listed 6, mostly in Phase II clinical trials

Conclusion

The genetic cell therapy of MTT mediates its effect through transfer of the normal myoblast nuclei that supply the complete human genome, in addition to just replenishing the aberrant gene(s). The replacement genes then transcribe to produce the necessary proteins or factors for genetic repair. Donor myoblasts also develop to supply significantly large numbers of normal myofibers to combat muscle degeneration and weakness of genetic or nongenetic origin.

Usage of the MTT inventions includes disease treatment, disease prevention, drug discovery, selection of superior cells and clones for therapy (Law et al. 2019), in addition to providing regenerative networks of mitochondria, ribosomes, sarcoplasmic reticulum, and other cell organelles.

It is through continual research and development that MTT will be fully harnessed to relieve human suffering, to improve quality of life, and to prolong the life expectancy of mankind. This unique platform technology, patented for its compositions, methods, and related medical devices of cell/gene therapies, promised to be of great social and economic values in world health and human services. Myoblast therapies should be implemented at the earliest by national health agencies for public health.

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Law PK. Myoblast therapy for mammalian diseases. WO9618303A1; AU5831199A; AU748997B2; AU4597696A; CA2183167A1; CNZL95192528.8 CN1146712A; CN1127343C;CN1477190A; DE P2116DE01; EP0743820A1; EP0743820A4; EP1407788A2; FR P2116FR01; GB P2116GB01; IE P2116IE01; SG74036A1; SG99279A1; SG99846A1; US2002031501A1; US6261832B1; US7341719B1

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23 Adipose Tissue-Derived Regenerative Cell-Based Therapies: Current Optimization Strategies for Effective Treatment in Aesthetic Surgery

Yusuke Shimizu, Edward Hosea Ntege, and Hiroshi Sunami

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Abstract

Mesenchymal stem cells (MSCs) are adult stem cells derived from several different tissues in the human body, such as adipose tissue. Adipose tissue is composed of mature adipocytes and a stromal vascular fraction (SVF). Fresh SVF cells are heterogeneous, containing putative MSCs (adipose tissue-derived stem cells; ASCs), progenitor cells, vascular smooth muscle cells or pericytes, and hematopoietic cells under uncultured conditions. The capacity to isolate SVF and ASCs from the readily accessible and abundant adipose tissue shows great promise as a crucial unlimited supply for numerous applications, including regenerative medicine and basic research for pharmaceutical discoveries. The SVF is used for immediate autologous applications during surgery, while purified

Y. Shimizu (✉) · E. H. Ntege
Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
e-mail: yyssprs@med.u-ryukyu.ac.jp

H. Sunami
Center for Advanced Medical Research, School of Medicine, University of the Ryukyus, Okinawa, Japan
e-mail: sunami@med.u-ryukyu.ac.jp

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or cultured ASCs are considered suitable for cell therapy owing to their inter alia proliferation capabilities, multilineage differentiation capacities, low immunogenicity, immunomodulatory properties, and various trophic properties. Moreover, ASCs can be used in long-term autologous and allogeneic applications. However, various studies have identified the need for improvement of the therapeutic potential of ASCs. With ethical and scientific controls and standardization of production and handling, as well as identification of acceptable modes of therapeutic applications, ASCs are envisaged to make a credible contribution to the clinical practice of the twenty-first century. Thus, different strategies for optimizing the performance of ASCs as a therapy are currently under investigation, including two treatment approaches: fresh, uncultured, unmodified, autologous SVF and ASCs. This chapter aims to review these two treatments as well as other clinical application optimization strategies for adipose tissue-derived regenerative cells.

Keywords

Adipose tissue · Aesthetic surgery · Cell therapy · Culture-expanded · Mesenchymal stem cells · Regenerative medicine · Stromal vascular fraction

Abbreviations

ASCs	Adipose stem cells
AT	Adipose tissue
AT-MSCs	Adipose tissue-derived mesenchymal stem cells
b-FGF	Beta-fibroblast growth factor
BM	Bone marrow
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CCL	Chemokine ligand
CD	Cluster of differentiation
CFU-F	Colony-forming unit fibroblast
cGMP	Current good manufacturing practice
cGTP	Current good tissue practice
CSF2	Colony-stimulating factor 2
ECM	Extracellular matrix
FDA	Food and Drug Administration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCT/Ps	Human cells, tissues, or cellular and tissue-based products
HLA	Human leukocyte antigen
HNF4 α	Hepatocyte nuclear factor 4 alpha
IFN-γ	Interferon gamma
IGF	Insulin growth factor
IL	Interleukin
ISSCR	International Society for Stem Cell Research
MCP	Monocyte chemoattractant protein

MHLW	Japan Ministry of Health, Labour and Welfare
MIP	Macrophage inflammatory protein
miR	Microribonucleic acid
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stromal cells
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factors
PMDAct	Pharmaceuticals and Medical Devices Act
RMAct	Act on the Safety of Regenerative Medicine
SAEs	Serious adverse events
SCs	Stem cells
SVF	Stromal vascular fraction
TCOM or $TcPO_2$	Transcutaneous oxygen measurement
TF	Tissue factor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Introduction

In aesthetic surgery, adult stem cells are considered the most promising cell types for cell-based therapies. These are rare undifferentiated cell populations that are small in number and reside among differentiated cells in the body's tissues or organs. Adult stem cells are capable of self-renewal and differentiation into specialized cell types. However, their ability to differentiate has led to controversy as some scientists suggest that their differentiation is limited to distinct cell types based on the tissue of origin; they are multipotent or unipotent cells (Vishwakarma et al. 2014). In contrast, there are repeated demonstrations of the vascular-associated stem cells, forming other cell types due to their multilineage differentiation capacity (Alt et al. 2020). Adult stem cells were discovered in 1909 as hematopoietic stem cells (HSCs) in the bone marrow (BM) (Giannoudis et al. 2013). They were later described in the 1970s by Friedenstein as a population of cells capable of osteogenesis in vitro (Friedenstein et al. 1970) as well as in vivo (Friedenstein et al. 1974). They were later identified as multipotent cells having characteristics of plastic adherence and multilineage differentiation capacity, referred to as colony-forming unit fibroblasts (CFU-F) (Afanasyev et al. 2009). CFU-F has since generated worldwide interest in both research and clinical practice, and in the early 1990s, they were termed "mesenchymal stem cells" (MSCs). MSCs have been widely studied and revealed to be very promising cell therapy products, albeit with some challenges (Caplan and Dennis 2006; Galipeau and Sensébé 2018). Various studies have consistently demonstrated MSCs as heterogeneous, nonclonal mixtures of multipotent stem cells, committed progenitors, and differentiated cells (Squillaro et al. 2016). Consequently,

the MSC nomenclature has been contested leading to the adoption of several other names, including “marrow stromal cells,” “multipotent stromal cells,” “mesodermal stem cells,” or “mesenchymal stromal cells.” Relentless discussions on the accurate description of the origin, developmental potential, and biological functions of MSCs have proposed “tissue-specific progenitor cells” or “medicinal signalling cells” as more appropriate terms (Caplan 2017; Robey 2017; Sipp et al. 2018; Caplan 2019; De Luca et al. 2019). Besides, recent reports suggest that MSCs arise from pericytes and can be isolated from almost every tissue that is vascularized, including BM (Wakitani et al. 2002), adipose tissue (AT) (Zuk et al. 2001), umbilical cord (Kern et al. 2006), dental pulp (Stanko et al. 2018), and skin (Ojeh et al. 2015).

A recent review (Ntege et al. 2020) highlighted that the past three decades of research on MSCs has presented encouraging preclinical findings on a wide range of disease models. This promising evidence for the therapeutic success of MSCs is based on the cell’s relative ease to isolate and expand in culture, as well as its multilineage differentiation capacity; immunomodulatory, anti-inflammatory, antimicrobial, and regenerative effects; homing and migration to injury sites; and safety profile in allogeneic transplantation, with only a few ethical restrictions (Gnecchi et al. 2008; Teixeira et al. 2013; Vizoso et al. 2017; Caplan 2019). However, some MSC-based cell therapies in advanced trials have produced mixed reports on clinical efficacy leading to skepticism regarding their effectiveness. Deciphering the key factors that influence these biological and pharmacological disparities could be paramount in promoting the desired clinical efficacies. Key factors significantly contributing to dissonance include variable tissue sources, protocols used for cell preparation, potency, the functionality of MSCs among tissue sources, culture and expansion levels, cell handling in the clinic, etc. (Raposio et al. 2017; Galipeau and Sensébé 2018; Mushahary et al. 2018). Tissue sources of MSCs such as BM and AT have been extensively studied in clinical trials (Moroni and Fornasari 2013; Galipeau and Sensébé 2018). Bone marrow MSCs (BM-MSCs) demonstrate significant potential in promoting tissue regeneration, preventing tissue ischemia, and modulating inflammation and immunity (Murphy et al. 2013). Although BM-MSCs were considered the most promising among the MSCs cell-based therapies, they have significant limitations in clinical practice (Galipeau and Sensébé 2018): (i) BM in humans is commonly obtained from the sternum and posterior iliac crest through surgical aspiration procedures. Aspiration is performed under general anesthesia due to the associated excruciating pain; there have also been a few fatal complications documented in the past (Marti et al. 2004). (ii) BM is composed of a small fraction of true multipotent stem cells (Ratajczak et al. 2014). Hence, a sufficient therapeutic dose of BM-MSCs requires large amounts of BM, which could exacerbate risky outcomes. (iii) In vitro expansion of BM-MSCs is susceptible to early senescence, which influences the treatment outcomes for some diseases described elsewhere (Chen and Tang 2019). In contrast, AT has long been considered a multifunctional organ that controls metabolic homeostasis, immunity, and other endocrine activities and is abundant in most individuals (Gimble et al. 2007; Coelho et al. 2013). AT (25 to 100 mL) can be harvested using minimally invasive liposuction procedures and yields orders of magnitude more adipose-derived MSCs (AT-MSCs) per unit

volume (Gimble et al. 2007; Miyagi-Shiohira et al. 2015). Compared to BM-MSCs, AT-MSCs are better at sustaining chromosomal stability during multiple rounds of cell division for expansion (Izadpanah et al. 2008). Therefore, because AT is an abundant source of MSCs for immediate and/or long-term utilization, it is a vital source for cell-based therapies (El-Sabbagh 2017). AT-MSCs are vascular-associated MSCs and can be categorized into two types: the stromal vascular cell fraction (referred to as AT-derived regenerative cells or SVF) and the adipose-derived stem cells (ASCs) (Alt et al. 2020). ASCs are obtained upon further processing of SVF (Zuk et al. 2001). Both cell populations are fundamentally different in content and morphology but possess similar biological attributes, such as the trilineage differentiation potential and the cell adhesion molecules' unique expression (Matsumoto et al. 2006; Glass and Ferretti 2019; Alt et al. 2020).

AT-Derived Regenerative Cells

SVFs and their potential to proliferate were first demonstrated in 1964 and 1977 by Martin Rodbell and Van and Roncari (Rodbell 1964; Van Robin and Roncari 1977). SVF has recently been described by Alt et al. as uncultured, autologous, fresh, unmodified, adipose-derived regenerative cells offered at the point of care in aesthetic surgery (Alt et al. 2020). Accordingly, SVF is another generic term that refers to an extract of vascular-associated MSCs and other cells from AT that lacks both adipocytes and connective tissue. It is also essential to know the distinction between SVF and nanofat. The latter is a mechanically emulsified fat tissue in a liquid form that presumably lacks connective tissue but contains cells of the stromal vascular fraction (Cohen et al. 2019; Alt et al. 2020). Specifically, SVF is a heterogeneous cell population composed of cells from endothelial, hematopoietic, and pericytic origin, among others. Cells of hematopoietic origin include granulocytes (15%), monocytes (15%), lymphocytes (15%), and stem/progenitor cells (<0.1%) (Bourin et al. 2013). The SVF comprises three major populations of stem/progenitor cells that are closely associated with small blood vessels, including endothelial progenitor cells, pericytes, and the supra-adventitial ASCs (Zimmerlin et al. 2013; Glass and Ferretti 2019).

Isolation of SVF from AT can be achieved through either enzymatic or non-enzymatic (such as purely mechanical) methods (Winnier et al. 2019). The ideal isolation method for a desirable therapeutic outcome should aim to yield sufficient numbers of viable cells from the smallest possible amount of AT in the shortest possible time and minimize the use of non-viable cells to avoid unwanted inflammatory reactions (Snyder et al. 2019). The enzymatic method is based on dissolving connective tissue and walls of vascular structures of AT by protease enzymes to release in situ stem cells. This is the most favorable method because it leads to significantly high cell yields with high viability scores. The developing mechanical isolation technologies are highly promising, i.e., Transpose RT/Matrase System (InGeneron Inc.) that can deliver high cell yields with high cell viability (Winnier et al. 2019).

SVF is helpful in plastic surgery for several reasons: (i) It has been repeatedly demonstrated that the SVF is safe and effective in regeneration medicine and tissue engineering (Gentile 2019; Gentile and Garcovich 2019; Gentile et al. 2014, 2020; Granel et al. 2015). Of note, however, several serious adverse events (SAEs) related to stem cell (SC) treatments have been published, highlighting concerns of limited reporting outside clinical investigations as described in the review by Alt and colleagues (Alt et al. 2020). In this review, the authors highlight SAEs such as the development of glioproliferative lesions in the spinal cord leading to a plethora of symptoms following intrathecal administration of putative MSCs, vision loss after intravitreal injection of autologous SVF for the treatment of age-related macular degeneration, and the development of the lethal human herpesvirus 6-related meningoencephalitis, myocarditis, and interstitial nephritis after allogeneic transplantation of SCs for chronic lymphocytic leukemia. In contrast, other reports have indicated a lack of serious safety concerns (Toyserkani et al. 2017); (ii) SVF has intrinsic abilities to adequately regenerate tissue without the need for more than minimal manipulation, stimulation, and/or genetic reprogramming of the cells; and (iii) tissue regeneration with SVF fulfils the criteria of homologous use as per the ethical, legal, and regulatory standards. SVF can induce angiogenesis in different target tissues, including AT (Oranges et al. 2019), bone (Solakoglu et al. 2019), and ischemic myocardium (Haenel et al. 2019). The effective application of SVF for angiogenic purposes fulfils the criterion for homologous use, an SC clinical application regulatory requirement. Thus, SVF is much more easily acquired, without the influence of cell separation or culturing factors that could impact safety and other regulatory requirements, useful in soft tissue reconstruction, and suitable for autologous treatments (Simonacci et al. 2017). However, to achieve sufficient amounts of SVF for therapeutic purposes, harvesting large amounts of AT may be required (Aronowitz and Ellenhorn 2013). Moreover, deciphering the mechanisms of action behind SVF tissue regeneration remains elusive without modification, a potential violation of the SC clinical application regulations.

Adipose-Derived Stem Cells

A population of ASCs was first successfully isolated and characterized upon further processing of human SVF by Zuk et al. (2001). ASCs are a relatively homogenous, plastic adherent, and culture-expanded population mainly containing pre-adipocytes and vascular-associated MSCs (Zuk et al. 2001). Most studies indicate that depending on the isolation method, ASCs account for 10–30% of the total SVF (Kokai et al. 2014; Glass and Ferretti 2019). Isolation of ASCs primarily involves enzymatic processing in compliance with current good manufacturing practices (cGMP) and the local and international SCs regulatory rules and guidelines for research and clinical application. ASCs are isolated from freshly harvested or cryopreserved AT or SVF based on well-established protocols that require further standardization (Choudhery et al. 2014; Ntege et al. 2020). Briefly, SVF is usually harvested from fresh AT using 0.25% trypsin and 0.1% collagenase type I or II

proteases under atmospheric conditions of 5% CO_2 at 37 °C. The SVF is then taken through a series of washing and sometimes erythrocyte lysis steps before being suspended in a growth medium such as high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-mycotic solution at 37 °C with 5% CO_2 and cultured for 24 h to produce the plastic adherent ASCs. Culturing involves changing the growth medium every 2–3 days until the ASCs reach approximately 80–90% confluence, suitable for further processing or downstream applications.

The isolated ASCs should be characterized before every therapeutic and basic research application. ASCs can be identified and verified through the expression of unique surface markers (immune phenotyping) in the undifferentiated state and the ability to undergo trilineage differentiation, i.e., osteoblasts, adipocytes, and chondroblasts, under in vitro conditions (Dominici et al. 2006; Yoshimura et al. 2006; Bourin et al. 2013). Relevant cell assays are also carried out to monitor viability and proliferation, as previously described (Riss et al. 2016). Phenotypic validation is part of the safety evaluation that ensures genuine MSC-related adverse event monitoring in clinical investigations. Although ASCs are distinct in surface marker profiling, a unique single marker that distinguishes these cells remains elusive. Therefore, it is recommended that phenotyping should use multicolor flow cytometry analysis with added viability markers to eliminate dead or apoptotic cells. The selected cell surface markers for the classification of ASCs may vary depending on various factors, including AT anatomical location, isolation protocols, and culture conditions (Raposio et al. 2017; Glass and Ferretti 2019). According to the International Federation for Adipose Therapeutics and Science (IFATS), a foundational ASC phenotyping should include at least two negative markers (e.g., cluster of differentiation (CD) 3, CD11b, CD14, CD31, CD45, CD79a, or CD19, vascular cell adhesion molecule 1 (VCAM-1; CD106), CD235a, HLA-DR, and the bone marrow-derived stem cell-associated marker STRO-1) and two positive markers in the same analysis (e.g., tetraspan protein (CD9), CD13, β1 integrin (CD29), CD44, α4 integrin (CD49d), intercellular adhesion molecule (ICAM-1; CD54), ecto-5′-nucleotidase or CD73, Thy-1 or CD90, endoglin (CD105), activated leukocyte adhesion molecule (ALCAM; CD166)) (Bourin et al. 2013). Moreover, some markers such as CD31, VCAM-1, and CD146 are indeterminate but can still be considered. ASCs express moderate levels of CD34, especially in the early stages of cell culture; however, the expression levels decrease upon further passaging (Maumus et al. 2011). A study on AT histology analysis demonstrated that CD34-positive cells are primarily associated with vascular structures (Traktuev et al. 2008). It is also suggested that there are multiple classes of CD34 antibodies that recognize unique immunogens. Therefore, the choice of the CD34 antibody can substantially influence the signal intensity detected in a given cell population. Additionally, a small number of the ASC cell population are probably CD31-positive capillary endothelial cells; a CD34+/CD31− cell population of pericytic origin may also be derived from AT (Johal et al. 2015). It has been proposed that the surface markers CD36 (fatty acid translocase) and VCAM-1 help distinguish ASCs from BM-MSCs as ASCs do not express VCAM-1 but are moderately positive for CD36

(Maumus et al. 2011; Pachón-Peña et al. 2011; Bourin et al. 2013). To further strengthen the specific characterization of ASC, additional markers were suggested by Bourin et al. (Bourin et al. 2013; Pachón-Peña et al. 2011). These include the positive expression of variable levels of neprilysin or CD10, adenosine deaminase complexing protein 2 or CD26, CD49d (very late antigen-4), CD49e (very late antigen-5), and melanoma cell adhesion molecule (MCAM) or CD146 markers due to donor or culture passage differences and the low expression (<2%) levels of negative markers: CD3, CD11b (macrophage integrin-1), CD49f (very late antigen-6), and podocalyxin-like protein.

ASCs are increasingly becoming the cell therapy products of choice in plastic surgery and other clinical applications (Gir et al. 2012; Moll et al. 2019; Tobita et al. 2011) because of their excellent proliferation and multilineage differentiation abilities; that is, they can differentiate into endodermal (Li et al. 2013; Lee et al. 2015), mesodermal (Latief et al. 2016), and ectodermal lineages (Kang et al. 2004). Moreover, unlike the SVF, ASCs are suitable for both autologous and allogeneic treatments. ASCs can be cultured, especially for autologous applications that require large cell therapeutic doses (Garcia-Olmo et al. 2009), and are preferred for allogeneic purposes as they exhibit low immunogenicity that results from a low expression of major histocompatibility complex class II molecules and T- and B-cell costimulatory molecules CD80, CD86, and CD40 in vitro (Zhang et al. 2015b). Culture-expanded ASCs have improved cell homogeneity, can be precisely identified, and are generated in enough and sufficient therapeutic dosages. These characteristics are paramount in attaining high reproducibility of clinical outcomes (Lv et al. 2014; Lopa et al. 2019). Moreover, numerous peer-reviewed scientific reports promote further interest in the use of ASC-based therapies. For instance, it has repeatedly been demonstrated that the application of ASCs into a new host tissue or microenvironment achieves the following: (i) the cells can stay, survive, and engraft in the new host tissue (Bai et al. 2011), (ii) integrate into and communicate by direct cell-cell contacts (Alt et al. 2019), and (iii) can exchange genetic and epigenetic information through secretomes such as exosomes (Alt et al. 2019). There is increasing evidence that the secretome of the vascular-associated MSCs, including dead or inactivated fragmented MSCs (Weiss and Dahlke 2019), is responsible for its biological and pharmacological properties, such as immunomodulation, immunosuppression, anti-inflammatory, anti-apoptosis, and angiogenesis (Teixeira et al. 2013; Vizoso et al. 2017; Caplan 2019). Several publications (Schinköthe et al. 2008; Park et al. 2009; Kupcova Skalnikova 2013; Salgado et al. 2010; Blaber et al. 2012; Kokai et al. 2014) report that the ASC secretome in particular has higher levels of vascular endothelial growth factor (VEGF-D) messenger ribonucleic acid (mRNA) and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), and fibroblast growth factor (b-FGF) that support angiogenesis and proliferation as well as higher levels of pro-inflammatory cytokines such as interleukins (IL) 8, 1β, 6, and 12, tissue necrosis factor (TNF-α), and interferon gamma (IFN-γ) that support recruitment and activation of innate and adaptive immune cells, fibroblasts, and other MSCs; anti-inflammatory cytokines such as IL-10, IL-13, and prostaglandin E2 that mediate immunosuppression;

chemokines such as IL-8, monocyte chemoattractant protein (MCP) MCP-1 (CCL-2), macrophage inflammatory protein (MIP) MIP-1α (chemokine ligand CCL-3), and MIP-1β (CCL-4) that promote migration of innate and adaptive immune cells, fibroblasts, and MSCs; adipokines such as leptin and higher levels of insulin-like growth factor (IGF) IGF-1, adiponectin, steroid hormones, resistin, and plasminogen activator-inhibitor 1 (PAI-1) for AT homeostasis; matrix proteins such as Collagen-1 for extracellular matrix (ECM) synthesis; matrix protease such as matrix metalloproteinase (MMP) MMP-1 and MMP-2 for ECM remodelling and cell transit; and many other putative paracrine factors (Park et al. 2009; Skalnikova 2013; Kokai et al. 2014).

The use of ASC secretome in aesthetic surgery is beyond the scope of this chapter. However, it is worth mentioning that emerging studies report the ASC secretome to be a promising candidate for cell-free therapy strategies (Wang et al. 2019). However, all these studies were limited to cellular and animal assays without much evidence from large-scale clinical trials (Xiong et al. 2020). The ASC secretome can be utilized in tissue regeneration, but only to a certain extent as a supplementary treatment, rather than as sole therapy for skin anti-aging therapy, dermatitis improvement, wound healing, scar removal, flap transplantation, bone tissue repair and regeneration, obesity prevention, fat grafting, breast cancer, and breast reconstruction. As mentioned above, the ASC secretome has desirable properties that render it clinically promising as a novel cell-free therapeutic strategy. Briefly, these properties include the following: (1) The ASC secretomes, such as exosomes, are naturally occurring secreted membrane vesicles released from cells with lower immunogenicity; (2) ASC-exosomes contain a broad repertoire of cargoes, including nucleic acids, proteins, and enzymes for modulating multiple cellular activities, acting in both immediate and remote areas in a paracrine manner; (3) ASC-exosomes comprise natural bimolecular phospholipid structures that provide sufficient stability to avoid biodegradation; and (4) ASC-exosomes can function as a carrier for themselves as well as a component uploaded in well-designed biomedical materials. However, there are challenges associated with the development of the ASC secretome in clinical applications, including (i) the inconveniences related to obtaining ASCs. The sources of ASCs as well as the separation and cultivation methods, medium composition and dosage, cell passage, cell fusion and viability, mycoplasma, and other microbial contamination should be tightly controlled to maintain reliable biological efficacy and produce a high-quality ASC secretome; (ii) the extracted ASC secretome can be of low purity and yield, as the current separation methods, including ultracentrifugation, exclusion, ultrafiltration, two-aqueous system, immunoaffinity, and polymer precipitation, have shortcomings such as being time-consuming, labor-intensive, and costly and having multiple overnight centrifugation steps; and (iii), finally, the application of the ASC secretome, especially in tissue regeneration, still requires more comprehensive research in the following aspects: (a) quality control. ASC-exosome utilization details, including the storage conditions, effective doses, concentrations, and treatment period, are all essential aspects. It is necessary to further explore suitable microenvironmental conditions or genetic engineering techniques to ascertain the

efficiency of ASC-exosome treatment. (b) The components and functions of ASC-exosomes are comprised of multiple bioactive components. These complex multi-component substances may produce diverse biological characteristics when used in practice. Therefore, a deep understanding of ASC-exosomes and their components is a priority in reforming ASC-exosomes to overexpress these components to maximize the therapeutic effect while reducing side or off-target effects. (c) Carrier peculiarity exploration ASC-exosomes are effective tools for cargo transportation of effective therapeutic agents with lower immunogenicity and toxicity. Furthermore, ASC-exosomes could also be uploaded to specific nanomaterials or hydrogel materials to promote skin repair. Engineering ASC-exosomes to be effective and safe requires a comprehensive understanding of their necessary components, including but not limited to membrane stability, architecture, and packaging of the interior components. (d) Large-scale clinical trials that can clearly reflect the ASC-exosome usage and their physiological levels *in vivo* (Hong et al. 2019). In fat transplantation, it is of great value to clinically explore whether the exogenous ASC-exosomes could be safely and effectively used for cell transplantation (Atesok et al. 2017; Xiong et al. 2020).

Current Regulatory Considerations for the Production and Clinical Application of SVF/ASCs

In 2016, the International Society for Stem Cell Research (ISSCR) published the revised global standards for stem cell research and clinical translation, where new guidelines for preclinical research, clinical translation, and practice were stipulated (Daley et al. 2016). These guidelines strongly emphasize the importance of high standards of cell processing and manufacturing, good manufacturing practice (GMP), in the preparation of cell-based therapeutics. Like other medical therapies, cell-based therapies in aesthetic surgery must fulfil the minimal requirements for use, including prioritizing safety and clinical efficacy. The production or handling of SVF and ASCs for therapy must adhere to the regulatory guidelines currently set by individual national governments and in accordance with the ISSCR and other international standards such as the Declaration of Helsinki, the cornerstone document on human research ethics. For instance, in Japan, the regulations for cell-based therapeutics and related clinical investigations are implemented by two authorities: the Pharmaceuticals and Medical Devices Agency (PMDA) and the Ministry of Health, Labour and Welfare (MHLW) (Konomi et al. 2015). Two new laws were enacted in 2014: The Act on the Safety of Regenerative Medicine (RM Act) and the Pharmaceuticals and Medical Devices Act (PMD Act) (Azuma 2015; Hara et al. 2014). All regenerative medicine products and related clinical trials are reviewed by the PMDA, an incorporated administrative agency established pursuant to the PMDA Act and approved by the MHLW. The RM act covers other clinical research submissions for approval or daily medical treatments that use unapproved regenerative medicine products. This ensures that stem cell products processed within

medical institutions or outside companies with appropriate licenses can be used for clinical research or medical treatment. The PMD Act defines regenerative medicine products as follows: (1) processed human or animal cells intended for either (a) the reconstruction, repair, or formation of the structure or function of the human (or animal) body (i.e., tissue-engineered products) or (b) the treatment or prevention of human (or animal) diseases (i.e., cellular therapy products) and (2) articles intended for the treatment of disease in humans (or animals) and that are genetically manipulated to express in human (or animal) cells (i.e., gene therapy products) (PMD Act Article 2(9)). The cabinet ordinance of the PMD act (Articles 1–2) further specifies the following three product categories as regenerative medicine products: (1) processed human cell products, such as adult stem cell products, (2) processed animal cell products, and (3) gene therapy products, which are products that introduce genes to cells that are already in the human body (in vivo) or have been extracted from but then transplanted back into the human body (ex vivo) (Azuma 2015). In the United States, regulation of cell-based therapeutics is by the US Food and Drug Administration (FDA). A published review article summarized FDA regulations (Jokura et al. 2018). SVF and ASCs are categorized under human cells, tissues, or cellular and tissue-based products (HCT/Ps) (Gir et al. 2012). The production of HCT/Ps must comply with the current good tissue practice (cGTP) requirements under Title 21 Code of Federal Regulations, Part 1271 (21 CFR Part 1271). The FDA defines HCT/Ps as articles comprising human cells or tissues that are produced for implantation, infusion, or transfer into a human recipient. The indispensable cGTP requirements strive to prevent HCT/Ps from introducing, transmitting, or spreading any communicable disease, as previously reported (Smith et al. 2013). HCT/Ps are regulated at two levels of risk: low and high levels of risk. The low level of risk involves regulation of HCT/Ps solely under Section 361 of the Public Health System Act when all the following criteria are met (Part 1271.10): (a) The HCT/Ps are minimally manipulated; (b) HCT/Ps are produced for homologous use only; (c) the HCT/Ps manufacture does not combine them with other article (s); and (d) the HCT/P does not have a systemic effect and is not dependent on the metabolic activity of living cells for its primary function, or the HCT/P has a systemic effect or is dependent on the metabolic activity of living cells for its primary function and is for autologous use. At this level, the FDA could sanction the HCT/P as an investigational new drug in clinical investigations, and a formal FDA approval process for the specific therapy is not required. A high level of risk considers HCT/Ps a drug, device, or biological product of more than minimal manipulation, for example, ex vivo expansion, combination with non-tissue components, or transduction of the cells. Such HCT/Ps are regulated under Section 351 of the Public Health System Act, and the affected SVF or ASCs are licensed and delivered for clinical use as drugs. Licensing is performed only after HCT/Ps have been proven to be safe and effective. However, in the developmental stage, such products may be distributed for clinical use only if the sponsor has an investigational new drug application in effect as specified by the FDA (Title 21, Code of Federal Regulations, Part 312).

Assessing the Therapeutic Potential of SVF/ASCs in Aesthetic Surgery

The encouraging therapeutic effects in animal models, including inter alia proliferation, potency, homing/migration, and paracrine actions, explain the rationale for the anticipated efficient translation of SVF/ASCs in regenerative medicine (Frese et al. 2016; Gnecchi et al. 2016). However, effective clinical translations require a careful and deep understanding of the factors that affect the performance and safety of SVF/ASCs. Several published reports indicate various factors that influence the therapeutic function of these cells, including age, sex, body mass index (BMI), AT origin, and donor comorbidity states such as diabetes, cell biology, and culture factors (Patrikoski et al. 2019). Glass and Ferretti (2019) recently published a review that highlighted the ability of stem cells to renew decreases with aging and is associated with the downregulation of SIRT-1 (silent information regulator 1) by microRNAs (miR). Indeed, miR-486-5p has been proposed to play a role in ASC replicative senescence (Kim et al. 2012b; Zhang et al. 2015a, b). Aging is associated with the downregulation of genes responsible for maintaining genomic integrity and chromatin remodelling, leading to functional attenuation and risk of neoplastic transformations (Chambers et al. 2007). Aging also influences the trilineage differentiation potential of MSCs, negatively affecting osteogenesis while enhancing adipogenesis, suggesting a differential growth factor expression in senescence that reduces bone morphogenetic protein (BMP) 2/4 and upregulates the expression of TGF-β (Sethe et al. 2006; Kim et al. 2012a). Besides, aging MSCs express higher levels of pro-inflammatory cytokines, which, in the absence of cytokine and chemokine receptors, results in a reduced ability to respond to injury (Bustos et al. 2014). The developmental origins of AT can affect ASC cell expansion, differentiation, and therapeutics. Notably, different visceral fat depots are heterogeneous, with significant differences in gene expression profiles and differentiation capabilities between ASCs derived from different fat depots. The depth of AT harvested at a particular anatomical location can also impact the ASC proliferation and adipogenic potential. For instance, ASCs from subcutaneous AT have increased proliferation and adipogenic capacities compared to ASCs of visceral origin (Patrikoski et al. 2019). The aforementioned conditions, including cell isolation and culture, can also affect ASC proliferation, differentiation, and paracrine function (Alharbi et al. 2013; Kim et al. 2016). Therefore, an in-depth investigation of the factors promoting patient-specific treatments, preparation of efficacious off-the-shelf treatments (allogeneic ASC treatments), and the optimization and standardization of isolation and culture methods for mass production as well as cell handling and delivery are essential to improve the clinical efficacy of ASC cell therapy.

Safety Concerns with the Use of Culture-Expanded ASCs for Cell Therapy

The ex vivo expansion of ASCs is essential in selecting relatively more homogenous cell populations that meet the standard criteria for identification and precise determination of therapeutic dosages to ensure high reproducibility of clinical outcomes (Lv et al. 2014). Notwithstanding, some reports highlight safety concerns regarding

in vitro expanded MSCs (Karnoub et al. 2007; Uccelli et al. 2008). It is indeed worrisome to some researchers that cell expansion outside their natural environment could increase the risk of genomic instability or altered differentiation potential, leading to serious adverse events such as tumors, teratomas, and severe immune reactions. The lack of counter immune surveillance and the influence of different culture induction factors have been explicitly fronted as likely factors of influence. Although genomic instability has been associated with the long-term culturing of cells, ASCs are reportedly genetically stable on multiple cell divisions even under xeno-free conditions (Grimes et al. 2009; Neri et al. 2017). Nonetheless, the risk of neoplastic transformations requires further studies as MSC cytokine features such as angiogenesis, cell migration, proliferation, renewal, epithelial transdifferentiation, and immunomodulation are potentially tumorigenic (Spaeth et al. 2013). MSCs can be attracted to sites of tissue injury as well as tumor microenvironments and differentiate into myofibroblasts known to promote tumor growth; such features are critical contributors to tumor aggression and invasiveness in breast lipofilling. Moreover, BM-MSCs specifically increase the tumorigenicity and invasiveness of breast cancer cells by inducing the de novo expression of CCL5. This chemokine acts in a paracrine manner to increase cell migration (in vitro) and extravascular translocation (in vivo) (Karnoub et al. 2007).

Similarly, induced de novo expression of the stromal cell-derived factor 1 (SDF-1) chemokine, also known as C-X-C motif chemokine 12 (CXCL12), has been reported in ASCs, which increased tumorigenicity and invasiveness, and was reversed by the inhibition of the corresponding receptors (Muehlberg et al. 2009). Among the additional reports supporting the possibility of malignant transformations, there are widespread reports on sarcoma formation in immunodeficient mice following injection of in vitro post-senescence-transformed ASCs (Rubio et al. 2005) and that, on the use of fetal calf serum in cell culture, present a risk for prion exposure (Halme and Kessler 2006). The former was retracted because, later, it was shown that the cells used in the transformation studies were cross-contaminated by cancerous cells that initially grew slowly in the presence of human MSCs (Torsvik et al. 2010), and the latter remains debatable. In contrast, ASC and ASC-conditioned supernatants have been shown to induce necrosis in a range of tumor cell lines in vitro and in vivo (Cousin et al. 2009). A recent study designed to evaluate fat transplantation as a supportive environment for tumor growth firmly concluded that it does not and that ASCs may even be suppressive (Tsuji et al. 2018). This school of thought has been supported in other published reviews on the clinical studies involving fat grafting for oncological breast reconstruction (Groen et al. 2016). Additional safety concerns arguably include an exhibition of highly pro-coagulant activity and even lethal effects upon infusion of ASCs in preclinical models (Tatsumi et al. 2013) and cases of peripheral microthrombosis, pulmonary embolisms, and even suspected cases of death in patients receiving ASC intravascular infusions (Cyranoski 2010). By and large, MSC products are generally considered safe in the clinic (Borakati et al. 2018). The IFATS has not yet reported ASC-based clinical studies associated with increased risk for adverse events/infusion toxicity (Toyserkani et al. 2017). Moreover, in a recent dose-escalation study, intravenous infusions of ASCs from

healthy donors were well tolerated by humans with up to 4×10^6 cells/kg body weight (Perlee et al. 2018).

Clinical Application of SVF/ASCs in Aesthetic Surgery

As summarized in Table 1, there has been a remarkable increase in the utility of SVF/ASCs in plastic and cosmetic surgery, with most MSC-based clinical trials conducted for a variety of diseases at phase I and II levels. A recently published review by Chu et al. (2019) highlighted a trend of year-by-year increase in the number of clinical trials using AT-MSCs starting in 2007 and peaked with a total of 187 in 2015. Also, other reports also indicated a total of 282 registered trials in late 2018; 22 (8%) utilized SVF, with 13 (5%) progressing to advanced phases (Patrikoski et al. 2019).

Soft Tissue Reconstruction

The application of SVF/ASCs in aesthetic surgery is common in trials involving the repair of soft tissue defects (Gimble et al. 2010; Alt et al. 2019). Soft tissue reconstruction remains one of the most significant challenges in plastic surgery. Defects in soft tissue are often caused by trauma and congenital diseases, such as Romberg disease and Poland syndrome, and as sequela to oncologic treatment. Among the various techniques employed to reconstruct such defects is the transplantation of autologous AT.

In the 1990s, Coleman et al. began treating soft tissue defects using transplanted fat, and the technique has been evolving for the last two decades (Coleman 1995, 1997, 2001, 2006; Coleman and Carraway 2002; Coleman and Saboeiro 2007). The technique has revolutionized soft tissue reconstruction, albeit with unpredictable outcomes due to high graft resorption rates resulting from lack of vascularization (Klinger et al. 2015). However, the use of ASCs has improved fat grafting outcomes, mainly due to the unique properties of the cells, including the relative ease of differentiation into AT, angiogenic capacity, and the ability to express and secrete multiple growth factors (Egro and Marra 2018). Studies on the impact of ASCs on fat transplantation have been reported. These include the following: (i) in 2006, a reported demonstration of cell-assisted lipotransfer (CAL) in boosting efficacy and reducing adverse effects of lipoinjection (Matsumoto et al. 2006); (ii) Rigotti et al. (2007) demonstrated the high therapeutic effects of lipoaspirates containing ASC treatments on degenerative, chronic lesions due to oncologic radiation; and (iii) multiple studies conducted on the condensation of ASCs resulting in increased interest in using fat grafting to revitalize stem cell-depleted tissues (Kølle et al. 2013; Kuno and Yoshimura 2015; Bellini et al. 2017).

In addition to their excellent regenerative abilities, the relative homogeneity and advances in cell production technology that significantly improve the purity of ASCs are critical to the improvement of therapeutic outcomes (Mazini et al. 2019). Thus, further optimization strategies that reduce risk related to manipulation and handling will lead to ASCs playing a primary role in the regenerative medicine of the twenty-

Table 1 Selected previous and current AT-MSCs cell-based clinical studies in plastic and aesthetic surgery

Disease	Trial phase	Cell type/route of administration	Autologous / heterologous	Mode of action	Findings	Reference /trial ID
Soft tissue regeneration						
Secondary-progressive multiple sclerosis	Phase I/II	ASCs/intravenous injections	Autologous	Homing to affected site	Safe and feasible in patients. No significant changes in safety parameters	Fernández et al. (2018)
Fingertip injury	Pilot study	ASCs/injection	Autologous	Regenerative potential	First time demonstration of the regenerative potential of liposuction aspirate fluid adipose-derived stem cells	Tarallo et al. (2018)
Breast augmentation	Phase I/II	ASCs/injections	Autologous	Fat graft enhancement	Circumferential breast measurement improvement and stability on follow-up; cyst formation in two patients	Kamakura and Ito (2011), Tiryaki et al. (2011), Wang et al. (2012), and Yoshimura et al. (2008), Davis et al. (2020), Turner et al. (2020)
	Unknown; staged cell-enriched fat transplantation	ASCs/injections	Autologous	Fat graft enhancement	Single session of stem cell-enriched tissue (SET) injections was safe and provides superior results compared to traditional fat grafting	
	Unknown; CAL treatment	ASCs/injections	Autologous	Fat graft enhancement	Significant postoperative volume increase and greatly improved breasts' contour. CAL	

(continued)

Table 1 (continued)

Disease	Trial phase	Cell type/route of administration	Autologous / heterologous	Mode of action	Findings	Reference /trial ID
					was safe and effective for breast augmentation	
Breast augmentation revision	Phase I/II; CAL treatment	ASCs/injections	Autologous	Fat graft enhancement	Preliminary results suggest CAL was suitable for the replacement of breast implants with no complications	Yoshimura et al. (2010)
Facial lipoatrophy (Parry-Romberg syndrome)	Unknown; CAL treatment	ASCs/injections	Autologous	Angiogenic effect	CAL was promising in decreasing the rate of fat reabsorption	Castro-Govea et al. (2012)
Facial augmentation	Unknown; CAL treatment	SVF/injections	Autologous	Fat graft enhancement	Subjective patient and surgeon satisfaction of the SVF-assisted fat grafting procedure due to superior results	Lee et al. (2012b)
Wound healing						
Diabetic foot ulcer	Phase I/II	ADSC-enriched fibrin gel	Autologous	Epithelialization, angiogenesis, proliferation, and collagen matrix formation		NCT03865394

Radiotherapy tissue damage	Phase I/II	SVF/repeated low-invasive computer-assisted injection	Autologous	Tissue regeneration	Exhibited progressive regeneration with systematic improvement or remission of symptoms in all evaluated patients	Rigotti et al. (2007)
Critical limb ischemia	Pilot study	ASCs/intramuscular injections	Autologous	Angiogenic effect	Digital subtraction angiography showed formation of numerous vascular collateral networks across affected arteries	Lee et al. (2012a)
Critical limb ischemia	Phase I/II	ASCs/intramuscular injection	Autologous	Angiogenic effect		NCT01211028
Scars or cutis laxa	Phase I/II	SVF/injection combined with laser therapy	Autologous	Tissue regeneration	Scars or cutis laxa	NCT03887208
Alopecia	Unknown	Transplantation	Unknown			NCT03427905
Facial rejuvenation	Unknown	Intradermal injection	Autologous			NCT03928444
Bone/cartilage regeneration						
Osteoarthritis	Pilot study	ASCs/intra-articular injections	Autologous	Healing and regenerate cartilage-like tissue	ASCs were safe and improved pain, function, and cartilage volume of the knee joint	Song et al. (2018)
Knee osteoarthritis	Phase III	SVF/intra-articular injection	Autologous	Healing and regenerate cartilage-like tissue		NCT03467919

(continued)

Table 1 (continued)

Disease	Trial phase	Cell type/route of administration	Autologous / heterologous	Mode of action	Findings	Reference /trial ID
Knee osteoarthritis	Phase I/II	Fat pad transplantation with ADSCs	Autologous	Regenerate cartilage-like tissue		NCT03014401
Knee Osteoarthritis	Phase I/II	ASCs/intra-articular injection	Allogenic	Regenerate cartilage-like tissue		NCT02784964
Vestibulodynia	Unknown	ADSC transplantation via lipofilling	Unknown			NCT03431779
Isolated articular cartilage defects	Unknown	ADSC-enriched acellular dermal matrix	Autologous	Regenerate cartilage-like tissue		NCT02090140
Degenerative disc disease	Phase I	SVF/injection	Autologous	Decrease low back pain	Demonstrated strong safety profile	Comella et al. (2017)
Calvarial reconstruction	Unknown	ASCs seeded in beta-tricalcium phosphate granules	Autologous	Bone regeneration	CT scans and clinical examinations revealed satisfactory outcome in ossification	Reviewed in Banyard et al. (2015)
Maxilla reconstruction	Case report	Microvascular flap with ASCs, beta-tricalcium phosphate, and bone morphogenetic protein 2	Autologous	Bone regeneration	Successfully produced ectopic bone using autoASCs in microvascular reconstruction surgery	Mesimäki et al. (2009)

first century (Cervelli and Gentile 2009; Cervelli et al. 2013; Mazini et al. 2019). The inability to inject high volumes of fat to improve fat graft survival rates led to the concept of pre-expansion of ASCs as portrayed in the following breast reconstruction and augmentation efforts: (i) Dr. Khouri's nonsurgical breast enlargement system, Brava™ (Khouri et al. 2000). The Brava system utilizes mechanotransduction principles to convert mechanical tension (by using low continuous vacuum distraction force to each breast) into growth-promoting signals, which creates an autologous 3D vascularized scaffold that can be used for grafting large volumes of fat. The first case series was published in 2000, and the authors showed a stable long-term increase in breast size by 55% (range, 15–115%) with no adverse events (ref). Since then, multiple studies have proven its efficacy and safety in both aesthetic and reconstructive settings. (ii) Zocchi and Zuliani (2008) published their 9-year experience of using Brava™ plus autologous fat grafting in 181 patients undergoing body contouring and/or breast augmentation (+/− reduction mammoplasty or mastopexy). The authors grafted a mean volume of 375 mL per breast with a 55% retention rate at the 1-year follow-up; (iii) Khouri et al. (2014) published a 9-year multicenter breast aesthetic experience of using Brava™ plus autologous fat grafting in 476 women. The augmentation group was grafted with a mean volume of 367 mL per breast per operation with an 80% retention rate (mean follow-up measurements taken at 9 months), and (iv) Khouri et al. (2015) also published a 7-year multicenter experience of using this technology to reconstruct the breasts of 488 women. The authors grafted a mean volume of 225 mL per breast per operation, leading to a mean breast mound volume of 375 mL per breast, but no graft retention data were presented. Compared to CAL or autologous fat grafting alone, the retention rates (78–80%) for the example presented by Khouri et al. are much higher (Khouri et al. 2014; Khouri et al. 2015). However, these studies lacked control groups to truly assess the impact of pre-expansion. Therefore, the favorable outcomes and high retention rates still require further investigation. Furthermore, randomized controlled studies are still lacking in the literature, and fundamental questions still need to be addressed.

Use of ASCs in Re-contouring Depressed Facial Lesions

In 2016, our group at the Department of Plastic and Reconstructive Surgery, University of the Ryukyus Hospital, conducted a clinical trial using culture-expanded ASCs to re-contour patients with depressed scars first time in Japan (Ntege et al. 2020). Our trial, a regenerative medicine provision plan (PB7150007) dubbed "Examination of treatment method for depressed lesions using cultured adipose tissue-derived stem cells (ADSCs)," was examined by a certified special committee for regenerative medicine and accepted by the Health and Welfare Bureau of MHLW (Examination of regenerative medicine provision plan records: https://japsam.or.jp/nintei/files/records/record-2018-02-20-nagoya.pdf). This was an investigator-driven, open-label, non-randomized, uncontrolled, phase I/II interventional study, which utilized the existing cell processing facility (CPF) at the University. The CPF was cGMP licensed under the guidance of PMDA and MHLW and employed highly skilled technical and clinical specialties to process

and for clinical application of ASCs. The trial was registered, approved, and conducted according to the local and international guidelines regulating the conduct of trials in human participants as described (Ntege et al. 2020). The trial recruited a maximum of 11 patients based on the underlying treatment concept and promising preclinical and clinical safety and efficacy data regarding the use of ASCs in treating a wide range of conditions. Participation was based on the following inclusion criteria: (a) age of 10 years and above male or female patients; (b) having a congenital or acquired depressed lesion indicated for fat grafting, including breast deficit as sequelae of breast cancer, traumatic tissue defects, hemifacial atrophy, facial muscle atrophy due to palsy, enophthalmos, and funnel chest; (c) depressed lesions with indications of secondary revision surgery and fat grafting; (d) cases of secondary revision surgery should have been due 3 months post-primary surgery; (e) willingness to provide informed consent; (f) history of at least 10 years of no serial illnesses; (g) willingness to live in Okinawa Prefecture at least 6 months following treatment; and, finally, (h) potentially eligible patients participating in another ASC study regarding, in particular, the development of an extraction system of safe and good-quality adipose-derived stem cells. The trial excluded patients who were pregnant, at risk of recurrence or metastasis of a malignant tumor, and under anti-cancer agent or immunosuppressive agent treatment or those who had local infections in or near the depressed lesion. The recruited patients who presented with depressed facial scars due to a wide range of causes, such as cancer treatment and Parry-Romberg syndrome, were treated and followed up according to the study protocol. Informed consent for the treatment and publication of outcomes was obtained from the patients before any procedures. The clinical evaluation of the therapeutic effects of ASCs was based on patient clinical assessments and interviews, as well as radiological and standard photographic investigations, including computed tomography scans for volumetric changes taken before and after treatment. Data on the aesthetic improvement of each trial subject and overall satisfaction were ranked from 1 to 10 (Table 2). The ASC treatment of facial defects followed standard techniques as described previously (Bellini et al. 2017). These included (1) harvesting of AT from a suitable donor site; (2) processing the lipoaspirate to eliminate cellular debris, acellular oil, and excess infiltrated solution followed by the production of ASCs; and (3) reinjection of the purified AT. Briefly, a small amount of fat was harvested in the University Hospital Surgery Theatres and aseptically transferred to the CPF for isolation and expansion of ASCs before injection into the depressed lesions, as reported elsewhere (Lewis 1991; Coleman 2006; Pu et al. 2008). The trial's anticipated outcomes included re-contouring assessment, cyst formation, incidence, severity, and duration of any other adverse events following 1 week and 1, 3, and 6 months after treatment.

In the present study, we present a report on the preliminary survey results for the first five trial subjects with mainly subjective patient and surgeon satisfaction level scores of the ASC treatment procedures summarized in Table 2. In particular, a case represented in Fig. 1 was a patient with malar deformity following treatment of maxillary sinus carcinoma. Overall, the findings suggested that the depressed scars

Table 2 Preliminary findings of a clinical study for the treatment of depressed facial lesions using ASCs

Patient age (years)/ gender	Presentation	Body mass index	Fat harvest procedure, amount, and site	Obtained and utilized no. of ASCs (per ML)	Aesthetic improvement score	Level of patient satisfaction
75/male	Right malar defect following maxillary tumor resection and radiotherapy	26.8	Excision, 24.2 g, lower left abdomen	2.0×10^7	10	10
12/male	Right temporal facial depression after cranial base tumor resection	18	Lipoaspiration, 9.9 g, lateral side of the thigh	2.0×10^8	8	9
42/male	Left post-scleroderma cheek depression	21.2	Lipoaspiration, 14.8 g, upper and lower abdomen	1.85×10^8	8	8
65/female	Left hemifacial atrophy due to Parry-Romberg syndrome	21.4	Lipoaspiration, 36.6 g, lower abdomen	8.4×10^7	9	10
65/female	Right temporal and forehead depression after cranial tumor resection	23.8	Lipoaspiration, 65.7 g, upper and bilateral aspects of the abdomen	7.2×10^7	9	10

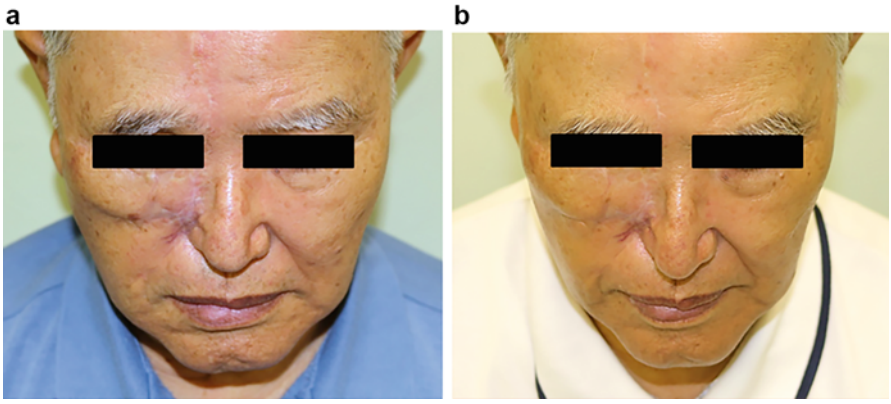

Fig. 1 Patient presenting malar deformity following a resected maxillary sinus carcinoma and treated using ASCs. (**a**) Preoperative image showing the malar deformity. (**b**) Two and half years of postoperative follow-up image shows a satisfactory outcome of a near-normal contour line. (*Reproduced with permission of the copyright to* Ntege et al. 2020)

were re-contoured to the patients' satisfaction, and no SAEs were reported even after 2.5 years of follow-up. These data represent the first milestone in the clinical evaluation of repairing soft tissue defects with fat grafts combined with culture-expanded ASCs in Japan. Fat grafts play an essential role in treating depressed or altered scars and other surgical problems (Bellini et al. 2017). Our trial experiences are in agreement with previous research findings (Raposio et al. 2017) and included challenges particularly related to the high cost of clinical-grade ASC production and sustainability of the GMP facility, the long time required to process and prepare cells for transplantation (3–5 weeks), and the variability in the autologous ASC product and use of biological reagents likely to influence clinical outcomes.

ASCs in Wound Healing

ASC cell therapy is gaining a prominent role in the future of wound healing. Although only a few clinical trials have been reported, the findings are promising and seem to suggest that ASCs in wound healing are effective and safe in the early stages (Bertozzi et al. 2017). Wound healing is a complex process involving inflammation, epithelialization, angiogenesis, proliferation, and collagen matrix formation. The process is carried out and regulated by numerous growth factors, cytokines, and chemokines, such as TGF-β; VEGF; PDGF; granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as colony-stimulating factor 2 (CSF2); the IL family; EGF; FGF; and TNF-α. The healing process's main obstacle is the reduction in the cytokines released by local inflammatory cells and decreased vascularization. Emerging evidence shows that ASCs effectively treat acute and chronic wounds even in clinical settings, but the exact mechanism of action is still under investigation. ASCs reportedly initiate or enhance tissue regeneration through two main

mechanisms, either by differentiation into skin cells or by secretion of paracrine factors that downregulate the inflammatory response (Ankrum et al. 2014). The primary mechanism is thought to be paracrine secretion, which leads to the subsequent differentiation of stem cells into endothelial cells, fibroblasts, or keratinocytes. Moreover, ASCs may modulate the "stem cell niche" by stimulating the recruitment of endogenous stem cells and promoting their differentiation along the required lineage pathway. ASCs also possess antioxidant effects during wound healing.

Published reports have exhibited encouraging results from the use of ASCs in treating chronic skin ulcers that often accompany peripheral artery disease, such as critical limb ischemia (CLI) (Marino et al. 2013; Bertozzi et al. 2017). Such chronic skin ulcers may not heal even after successful revascularization and often require special treatment. Moreover, several studies have adopted an approach based on the concept of improving the vascularization of ischemic limbs so that perfusion increases sufficiently to promote wound healing, reduces pain at rest, and allows limb salvation (Lawall et al. 2010). In these studies, ASCs were delivered mainly using multiple intramuscular injections and intra-arterial or intravenous administration. After cell implantation, perfusion was increased in the treated limbs, as confirmed by improvements in the ankle-brachial index, transcutaneous oxygen measurement (TCOM or $TcPO_2$), rest pain, and pain-free walking distance, with a reduction in amputation rates. Furthermore, Raposio et al. unpublished pilot study successfully treated seven patients presenting with ischemic ulcers of the lower limb with topical ASC administration. All patients were affected by either diabetes, peripheral artery disease, or both and were candidates for amputation. All patients were completely healed, with or without undergoing percutaneous transluminal angioplasty, and, hence, avoided limb amputation.

Moreover, both laser Doppler flowmetry and $TcpO_2$ values showed perilesional improvements in oxygenation and perfusion during the follow-up, validating the effectiveness of ASCs in inducing angiogenesis. The same group also reported successful treatment of 21 CLI cases using a combination of ASCs and PRP. Compared with the 31 CLI patients in the control group treated using standard wound care, the experimental group showed a significantly higher wound closure rate, resulting in faster recovery. In 2007, Rigotti et al. also demonstrated for the first time the ability of AT-MSCs as a means of regenerating the sequels of radiotherapy. The team showed that an injection of AT in severe radiation lesions (Late Effects Normal Tissue Task Force-Subjective, Objective, Management, Analytic (LENT-SOMA) scores 3 and 4) in 20 patients permitted angiogenesis and improved tissue hydration. Using this technique, regeneration of the radiation dermatitis zone was observed, which enabled simple reconstructions by split skin grafting instead of the usual, more debilitating reconstructions (Rigotti et al. 2007). By producing trophic support, the MSCs facilitated angiogenesis and reduced tissue inflammation achieving the desired clinical success (Shukla et al. 2015). Daumas et al. also successfully applied this treatment to patients who have scleroderma, an autoimmune pathology leading to tissue fibrosis and microangiopathy clinically referred to as Raynaud syndrome (Daumas et al. 2013).

Bone Reconstruction

Although fractures heal without complications, it is estimated that up to 10% may result in delayed or non-union (Thomas and Kehoe 2020). Besides, bone defects caused by traumatic and non-traumatic events such as infection, tumor resection, and skeletal abnormalities may not heal if the defect is large and the biological and mechanical environments are unfavorable (Giannoudis et al. 2007). Bone regeneration is a complex physiological process that is involved in continuous remodelling throughout adulthood. However, bone regeneration in such complex defects is sometimes challenging (Tajima et al. 2018).

Consequently, several techniques have been developed to reconstruct bony defects, including autografts, allografts, xenografts, and alloplasts. Although each technique has limitations, an autograft is considered the gold standard for treating non-unions, bone defects, and other causes of localized deficiencies in bone stock regardless (Campana et al. 2014). Autografts may be insufficient in treating bone union or healing of a large bone defect partly because of a lack of viable cells for osteogenesis and paracrine signalling that can sufficiently simulate the bone microenvironment (Fillingham and Jacobs 2016; Lee et al. 2019). ASCs can improve the efficacy of autografts through their demonstrated differentiation abilities toward the osteogenic lineage in vitro and great potential for bone regeneration in vivo (Mizuno et al. 2012). In addition, ASCs have been found to secrete many kinds of growth factors for tissue regeneration (Blaber et al. 2012). Experimental procedures based on ASCs have been on the rise, leading to increased amount of scientific data on ASCs and an extremely wide number of preclinical studies confirming their bone regenerative potential in vivo. Nonetheless, only a few controlled clinical trials aimed at assessing the efficacy and safety of ASCs in patients with bone-related disorders have been concluded and published, and only a few others are currently being carried out (Table 1).

Cross-References

- ► Advances, Opportunities, and Challenges in Stem Cell-Based Therapy
- ► Considerations for Clinical Use of Mesenchymal Stromal Cells
- ► Human Mesenchymal Stem Cells: The Art to Use Them in the Treatment of Previously Untreatable
- ► Mesenchymal Stromal Cells for COVID-19 Critical Care Patients
- ► Sources and Therapeutic Strategies of Mesenchymal Stem Cells in Regenerative Medicine

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Molecular Signature of Stem Cells Undergoing Cardiomyogenic Differentiation

24

Kavitha Govarthanan, Piyush Kumar Gupta, Binita E. Zipporah, Vineeta Sharma, M. Rajasundari, and Khawaja Husnain Haider

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K. Govarthanan (✉)
MK BHAN Young Research Fellow, Centre for Cardiovascular Biology and Disease, Institute for Stem Cell Sciences and Regenerative Medicine (inStem), Bengaluru, Karnataka, India
e-mail: kavithag@instem.res.in

P. K. Gupta
Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, Uttar Pradesh, India

Department of Biotechnology, Graphic Era Deemed to be University, Dehradun, Uttarakhand, India

B. E. Zipporah · V. Sharma
Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamilnadu, India

M. Rajasundari
Centre for Advanced Research (Stem Cells), Government Stanley Hospital, Chennai, Tamil Nadu, India

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia

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Abstract

Cardiomyocytes (CMs) are mitotically inactive but metabolically highly active cells that constitute a major population in the heart. The cumbersome isolation and purification protocols combined with the difficulty in their in vitro propagation as a primary culture remain the major impediments in their biological characterization. Therefore, understanding the molecular events that drive the successful differentiation of stem cells to become morphofunctionally competent CMs via advanced cellular technology is essential to better manipulate them for human applications. Cardiomyogenic differentiation of stem cells is relatively inefficient with a limited success rate due to the complexity of the molecular circuit involved therein. Akin to the embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) have vast prospective to give rise to any type of cell from the three germ layers without ethical concerns. Mesenchymal stem cells have limited differentiation potential toward cardiomyocyte lineage. Given their developmental aspects, the epigenomic signature often directs the cell's function which in turn governs their expression profile. Such epigenetic mechanisms include DNA methylation, histone core modifications, and miRNA regulations, which are associated with gene expression or silencing in the cell's progression toward a CM's fate. This chapter elucidates in-depth the regulatory process underlying CMs' differentiation at the genetic as well as epigenetic levels via the modulation of chromatin architecture. Specifically in this chapter, we will elaborate on the gene circuit and their expression profile in the various stem cells, heading toward cardiomyocyte lineage under different culture technologies from 2D, 3D, and even up to the single-cell level.

Keywords

Cardiomyocytes · Differentiation · Three-dimensional · Microarchitecture · Micropatterning · Organoids · Scaffold · Stem cells · Tissue engineering

Abbreviations

5-Az	5-Azacytidine
AhR	Aryl hydrocarbon receptor
AP	Action potential
BIO	6-Bromoindirubin-39-oxime
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CaMKs	Calmodulin kinases
CaMs	Calcium calmodulins
CBP	CREB binding protein
ciNSCs	Chemically induced neural stem cells
CSCs	Cardiac stem cells
Dsh	Dishevelled
EBs	Embryoid bodies
ECMs	Extracellular matrices
ERK-2	Extracellular signal-regulated kinase-2
GSK-3β	Glycogen synthase kinase-3β
HDACs	Histone deacetylases
hESC	Human embryonic stem cell
HMTase	Histone-lysine methyltransferase
IL-1Ra	IL-1 receptor antagonist
iPSCs	Induced pluripotent stem cells
JNK	c-Jun N-terminal kinase
LSD1	Lysine-specific demethylase 1
LVEF	Left ventricular ejection fraction
MEF	Mouse embryonic fibroblasts
MEF-2	Myocyte enhancer factor-2
mEpiSC	Mouse epiblast stem cell
mESC	Mouse embryonic stem cell
NaB	Sodium butyrate
NPC	Neural progenitor cell
NSCs	Neural stem cells
PCP	Planar cell polarity
PDK1	Phosphoinositide-dependent kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
pNSC	Primitive neural stem cell
PSCs	Pluripotent stem cells
ROCK	Rho-associated coiled-coil-containing protein kinase
SAHA	Suberanilohydroxamic acid
SHF	Second heart field
SIRPA	Signal-regulatory protein alpha
SkMs	Skeletal myoblasts
SRF	Serum response factor
TGF-β	Transforming growth factor-β
TMRM	Tetramethylrhodamine methyl ester perchlorate

TSA	Trichostatin A
Tzv	Thiazovivin
VPA	Valproic acid
Wnts	Wingless and Int-1 protein

Introduction

Cardiomyocytes (CMs) are the complex structural and functional unit of the heart, executing the vital function of transporting nutrients and fostering all the residual cells and organs in the body. CMs are specialized muscle cells with slow-cycling potential (so slow that they have always been considered as terminally differentiated cells that do not proliferate); hence, any damage to the myocardium will be irreversible (Allen et al. 1979; Yutzey 2017). With a severe shortage of humanized model systems for understanding the fundamentals of cardiomyocyte differentiation, non-human primates are often used as an experimental model for understanding the characteristic feature of CMs. The intricacy in understanding the biology of CMs is mainly due to their structural complexity and their correlated functional events, respectively (Ahuja et al. 2007). As a result of the advancement in cell culture techniques, few molecular details about CMs have been divulged during the last two decades. However, the mechanistic understanding of their metabolic, molecular, and physiologic is still being pursued. In this regard, stem cells of non-primate and human origin have been employed in several in vitro studies to decode the basic information about CMs. However, the molecular signaling involved therein has not been completely unveiled, which severely hampers the utility of CMs for cell-based therapy.

Knowledge about the characteristic feature of CMs is crucial to unveil in-depth details about pathophysiological aspects, cardiac disease manifestations, and its management. Thus, deciphering the understanding of pathological features of heart disease may warrant the development of novel pharmacological interventions for managing disease progression and treatment. This chapter will shed light on the so-far understanding of the mechanistic details of eagerness and the challenges prevailing in the dynamic area of cardiovascular research.

Basic Structure of Cardiomyocytes

The human heart consists of three layers, namely, the pericardium, myocardium, and endocardium, from outside inward. Grossly, the heart is divided into four chambers: two atria and two ventricles. The chief cell type present in the myocardium is CMs, which contains principal components such as sarcolemma, contractile elements (sarcomeres), and T-tubules participating in the excitation and coupling events, thereby facilitating the contraction and relaxation function of the heart (Woodcock and Matkovich 2005). Interestingly, CMs also have the sarcoplasmic reticulum that regulates the contractile element function and a large number of mitochondria due to

its aerobic metabolism dependence compared to skeletal muscle cells. Sarcomere consists of cardiomyocyte-specific proteins, namely, actin and myosin, performing cross-bridging and brings about the contraction and relaxation function of the heart muscle (Walker and Spinale 1999). Sarcomeres also possess regulatory functional proteins, i.e., troponin and tropomyosin, to facilitate the cross-bridging of actin and myosin filaments. Adjacent CMs are integrated via intercalated disks, which in turn consist of specialized intercellular junctional complexes including zonula adherens, desmosomes, and gap junctions (Walker and Spinale 1999; Molnar and Gair 2013). These junctional proteins predominantly participate in relaying mechanical and electrical coupling process, thereby conducting the electrical impulses throughout the different layers of the myocardium.

The most prominent tissue architecture of the human heart is preserved and maintained via the junctional component performing the adhesive interactions modulated by the cadherin family of proteins (Li et al. 2013). Therefore, a dysregulated expression of these junctional interactive components often leads to cardiovascular diseases (CVDs) (Thiriet 2019). Gap junction is the chief component of the intercalated disks, very crucial and often associated with CVDs. The primary function of intercalated disks is to facilitate the CMs' synchronous contraction as a syncytium via rendering electrical coupling potential as exhibited by CMs. Dysregulation of the gap junction proteins is often directly related to the CVDs pertaining to electromechanical dysfunction and arrhythmias (Manring et al. 2018). Another specialized structure, Purkinje fibers, is embodied in the conduction system and primarily aids in relaying the electrical signals spontaneously throughout the myocardium, thereby regulating the heart rate and rhythm.

Atrial and Ventricular Cardiomyocytes

Under the light microscope, the myocardium is observed to portray special features of atrial and ventricular CMs. The atrial walls are thin, and atrial CMs were smaller, leaner, and more elongated than the ventricular CMs. In humans and rodents, the light microscopic view shows a rectangular-shaped cell exhibiting a cross-striation pattern with alternating light isotropic (I) and dark anisotropic (A) bands, respectively. A typical mature cardiomyocyte has specialized cytoskeletal structure myofibrils, known as the contractile apparatus of a cardiomyocyte (Sweeney and Hammers 2018). Furthermore, these organized repeated subunits of myofibrils form a sarcomere, the functional unit of CMs. A mature sarcomere thin filaments (sarcomeric actins, troponins, tropomyosins) and thick filaments (myosin heavy and light chains) and associated proteins, such as myosin binding protein C, Titin, Z-lines, and M-lines. Some of the proteins are so specific for the heart that they are being exploited as cardiac-specific biomarkers (Haider and Stimson 1994, 1999). Thus, the complexity in the maturation is mainly due to the proper assembly and alignment of multimeric protein units in a well-organized manner (Pollard et al. 1974). Any defect or improper alignment in the arrangement of these protein units

generate functionally compromised CMs. Therefore, it is evident that the constitution and shape of the CMs are correlated directly to their functional maturity (Rommel and Hein 2020). It is believed that the human CMs are mostly mononucleated, whereas the rodent CMs exhibit the propensity of a bi-nucleated state under light microscope. Moreover, CMs nuclei show granulated chromatin structure with one or two nucleoli.

CMs are connected by intercalated disks forming a double membranous tight structure bound together by desmosomes and connected by gap junctions (Sun and Kontaridis 2018). This crucial junctional point serves smooth relaying of impulses from one cell to the adjacent cell, thus leading to spontaneous impulse conduction and synchronous contraction and relaxation. Histologically observed, ventricular myocytes are predominantly thick myofibrillar units with linear, broader, and denser T-tubules to perform the forceful contractions, while atrial myocytes are comparatively thinner. However, calcium signaling in both ventricular and atrial myocytes is similar but with increased calcium buffering efficiency in atrial myocytes (Smith and Eisner 2019). Likewise, the ventricular myocytes' sarcolemma is rich in ion channels which further aids in the longer-duration action potentials and effective refractory periods necessary for conducting the impulses.

Stem Cells and Cardiac Regenerative Therapy

Stem cell-based therapy has emerged as an attractive strategy for cardiac regeneration and repair. From among the various available options, embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) are endowed with unrestricted differentiation potential into almost all types of cells from the three germ layers (Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009), and hence, they possess huge potential to repopulate the infarcted heart (Tomescot et al. 2007). However, pluripotent stem cells (PSCs) are inherently teratogenic which render them unsuitable for routine clinical applications (Friedman and Moore 1946; Stevens 1967a, b; Damjanov and Solter 1974). Extra-embryonic tissue-derived stem cells, i.e., umbilical cord or placental tissue-derived stem cells, are endowed with restricted differentiation potential; however, their paracrine activity and immune-modulatory potential make them suitable for clinical use (Kim et al. 2013; Ullah et al. 2015; Rohban and Pieber 2017). Adult stem cells, i.e., bone marrow-derived mesenchymal stem cells (BM-MSCs), resident cardiac stem cells (CSCs), skeletal myoblasts (SkMs), etc., have been extensively studied and characterized in vitro as well as in preclinical studies either naïve or after genetically modulation (Haider et al. 2004a, b; Lei et al. 2005; Jiang et al. 2002; Haider et al. 2008; Lei et al. 2011; Elmadbouh et al. 2011; Takamiya et al. 2011) and have advanced to clinical trials (Haider et al. 2004a, b; Han et al. 2019; Sid-Otmane et al. 2020). Innovative cellular manipulating technologies using CRISPR/Cas 9 were employed to engineer stem cells for editing their characteristic profile supporting cardiac repair. This has become a prominent possible alternate option as a next-generation treatment for cardiac diseases up to the level of personalized therapy (Valenti et al. 2019; Nguyen et al. 2019).

Embryonic Stem Cells and Their Derivative CMs

ESCs are derived from the embryo proper or the inner cell mass of the blastocyst during the early embryogenesis. They are non-immune-privileged cells and, therefore, can be used for regenerative therapy only, subjecting the recipient to long-term immunosuppression (Pearl et al. 2012). Experimental studies have shown that a conducive milieu is required to modulate ESCs' cardiomyogenic and vasculogenic differentiation in vitro culture and post-engraftment (Leitolis et al. 2019; Jones and Wagers 2008; Bratt-Leal et al. 2009; Rufaihah et al. 2007, 2010). The provided milieu stimulates the appropriate signaling pathways directing the cell differentiation toward cardiovascular lineage, respectively. Among various signaling pathways, transforming growth factor-β (TGF-β) signaling is essential in mediating the cardiac differentiation of ESCs (Wolling et al. 2018). Some other signaling pathways involved include the activin pathway that facilitates mesoderm formation followed by bone morphogenetic proteins (BMP) for the further commitment to cardiac lineage. The timeline for activin A and BMP-4 supplementation for cardiac induction is crucial to achieve a higher percentage of beating CMs in vitro (Brand 2003; Wagner and Siddiqui 2007; Liu and Foley 2011; Brade et al. 2013; Sun and Kontaridis 2018). However, the obtained CMs are immature, and hence, further manipulation is required to facilitate their maturation (Veerman et al. 2015). To this end, researchers maintained the beating ESC-derived CMs in vitro culture for their maturation. Further manipulation in culture, such as three-dimensional (3D) matrices or scaffold and electrical stimulations, hastens the process of their maturation (Scuderi and Butcher 2017). The maturation of CMs is characterized by increased sarcomere banding and alignment, myofibril formation, MYH6/7 isoform switching, and spontaneous beating action potential similar to the adult CMs (Jiang et al. 2018). A detailed account of the strategies to derive cardiac lineage-specific cells from PSCs has been elegantly reviewed by Le and Hasegawa (2019).

Since the pioneering study of Caspi et al. (2007) which showed that transplantation of ESC-derived CMs could improve cardiac function in rodent model of myocardial infarction, various research groups have reported their therapeutic benefits in other experimental animal models (Caspi et al. 2007). For example, human ESC-derived CMs were successfully engrafted into the macacue heart and survived after 4 weeks of transplantation (Liu et al. 2018). In the porcine model, the engrafted ESC-derived CMs were found electromechanically coupled with the host CMs and thereby improved global heart function. ECM-derived CMs transplanted into the rodent heart model increased left ventricular ejection fraction (LVEF) (up to 40%) and reduced spontaneous or induced ventricular arrhythmias (Liew et al. 2020). In an experimental non-human primate model, ECM-derived CMs successfully survived and electromechanically coupled with the host myocardium after transplantation. The authors also reported significant re-muscularization in the infarcted heart (Weinberger et al. 2016). Unfortunately, the recipient animals developed ventricular arrhythmias which were not observed in the rodent model studies. Similar observations have also been reported by other research groups (Romagnuolo et al. 2019). Although the exact mechanism of increased arrhythmia was unclear, it was ascribed

to the cell graft size and its distinct stage of development and diverging heart morphology between the experimental animal models. In a nutshell, the application of ESC-derived CMs is very promising in the aspects of re-muscularization in the infarcted hearts; however, the issue related to arrhythmogenicity and teratogenicity needs to be addressed before it can be advanced to the clinics for human applications.

iPSCs and Their Derivative Cardiomyocytes

Yamanaka's landmark discovery of inducing pluripotency in normal somatic cells using a cocktail of four transcription factors, i.e., c-Myc, Oct3/4, Sox2, and Klf4, has given new impetus to stem cell research and has extended it's scope to include post-mitotic cells/slow-cycling cells for possible use in cell-based therapy (Takahashi and Yamanaka 2006). A subsequent study provided a comparison of the efficiency of two distinct sets of transcription factors, i.e., c-MYC, OCT3/4, and SOX2 and the other set with KLF4, or OCT3/4, SOX2, NANOG, and LIN28, to demonstrate successful conversion of human fibroblast into iPSCs using two sets of transcription factors, respectively (Schmidt and Plath 2012). Since the publications of these reports, various research groups have researched to optimize reprogramming protocol for its efficiency and safety for use in humans (Ibrahim et al. 2016). This included obtaining iPSCs employing different sets of transcription factors, using non-integrating viral vectors or non-viral vectors to alleviate insertional mutagenesis due to integrating viral vectors, and reducing genomic instability or genetic aberrations (Thomas et al. 2003). Later, protocol based on the introduction of miRNA and replacement of transcription factor with potent small molecule inhibitors has been reported to achieve safe and increased efficiency of reprogramming (Anokye-Danso et al. 2011; Higuchi et al. 2015a, b). These genetic modulation protocols have also been combined with advanced culture techniques for a more rapid and economical generation of iPSCs. On the same note, somatic cells from various tissue sources have been used to overcome the influence of their residual epigenetic memory and transcriptional perturbations after reprogramming to address its safety aspects before moving to clinical settings.

IPSCs have been studied for their myocardial reparability in experimental animal models of myocardial damage (Ahmed et al. 2011a, b; Buccini et al. 2012; Rajala et al. 2011). The transplanted cells successfully engrafted after intramyocardial delivery and improved global cardiac function. However, iPSCs' transplantation was not without the unwanted effect of tumorigenicity that was observed in at least 33% of the animals treated with iPSCs, thus raising serious concerns about their safety for use in humans (Ahmed et al. 2011a, b).

To overcome the undesired unbridled differentiation potential of iPSCs, various strategies have been developed. One of these strategies is the administration of iPSC-derived CMs, smooth muscle cells, or endothelial cells, which have shown successful engraftment at the damaged site in experimental animal models of myocardial damage. The successful engraftment of the delivered cells led to improved left

ventricular function in a murine model of myocardial infarction (Rojas et al. 2017). It is pertinent to mention that the purity of the genetically enriched iPSC-derived CMs was 99%, which matured post-engraftment, expressed cardiac-specific markers, and showed sarcomeric structures by 28 days after transplantation. No tumors were observed in any of the iPSC-derived cardiomyocyte-transplanted animals. Various other research groups have also reported similar data (Guan et al. 2020; Jiang et al. 2020). The transplanted cells also developed functional and electrical integration with the host CMs (Higuchi et al. 2015a, b). All these molecular and cellular changes help in the restoration of normal oxygen consumption in the infarcted heart (Ishida et al. 2019). Therefore, it is well-known that the high rate of re-muscularization and higher rate of donor cell engraftment at the injury site are essential for better prognosis. Besides strategies to enhance donor cell survival in the hostile environment of the infarcted heart, increased angiogenic potency of transplanted cells is considered important to enhance regional blood flow.

Researchers have endeavored to reprogram fibroblast directly into beating cardiac-like myocytes in vitro using defined transcription factors such as GATA-4, HAND2, MEF2C, and TBX5. Transplantation of rodent fibroblast-derived CMs has resulted in the scar size reduction, followed by enhanced LV function (Ieda et al. 2010). However, more in-depth studies are required to address the reproducibility of this reprogramming method besides this methodology to be equally useful for reprogramming of human fibroblasts. Furthermore, the retroviral-mediated reprogramming of human fibroblast should be carefully addressed for the infection recurrence, arbitrary genomic integration, tissue-specific homing, inflammatory reaction, etc. before considering for human therapeutic purposes.

Adult Tissue-Derived Stem/Progenitor Cells

Adult tissue-derived stem cells have been extensively characterized during in vitro, preclinical, and clinical studies, which suggests that the adult stem cell-based regenerative approaches successfully mitigate the myocardial damage and attenuate its progressive deterioration. Three of these cell types have already advanced to late-phase clinical trials. Embryonically, the human heart is derived from the mesoderm during gastrulation phase. Hence, it is logical that the cells having potency to generate mesoderm-derived tissues and organs could be a more relevant and suitable source for regenerative and tissue engineering approaches for cardiac repair. The occurrence of non-ESCs in the extraembryonic tissue, derived CD34+ HSCs differentiating into various blood cells, and the less differentiated MSCs. Additionally, the adult tissue-derived MSCs such as BM-derived MSCs, adipose tissue-derived MSCs, and the resident cardiac stem cells are also currently considered for cardiac cell-based therapy (Han et al. 2019). From the long list of the available adult tissue-derived stem cells, MSCs originating from the mesoderm have remarkable properties such as non-teratogenicity, immune-modulation, availability without moral or ethical issues, ease of expansion in vitro, and paracrine activity. Based on extensive research, paracrine action of MSCs is considered as one of the prime contributing

mechanisms in myocardial repair (Sid-Otmane et al. 2020). The released trophic factors ameliorate the cardiac tissue injury by promoting migration and retention of the intrinsic stem cells into the injured myocardium for repair and regeneration. This chapter will further shed light on MSCs' secretome including extracellular vesicles as a novel therapeutic option for cardiac tissue engineering and regeneration.

Molecular Profiling of Cardiomyocytes

CMs are considered as one of the highly specialized cell types in the body as they are mitotically inactive but metabolically highly active. As discussed earlier, iPSCs have the potential of generating functional CMs and, therefore, offer an exciting renewable cell source for cellular replacement therapy in the heart. This requires the development of an optimized protocol to generate homogenous population of cells, which is highly critical for the successive regenerative therapy. Therefore, understanding both upstream and downstream molecular regulatory pathways toward cardiomyogenesis is mandate for channelizing the scaling up process with utmost reproducibility concerns for transplantation process. This chapter highlights the protocols and molecular differentiation pathways that efficiently favor cardiomyocyte lineage. The time scale of induction of lineage-specific transcription factors and genes governing differentiation into cardiomyocyte lineage will also be discussed in this chapter. Collectively, this information provides the understanding of cardiomyocyte biology at both basic and advanced molecular level and thus may provide a new clinically relevant source of cells for therapeutic purpose.

Cardiomyocyte Lineage Commitment and Differentiation

Differentiation of stem cells into CMs has prerequisites for some key events, such as commitment to cardiomyocyte lineage followed by the induction of specific markers' expression to further facilitate the differentiation process. Hence, the knowledge of embryonic heart development is of utmost significance and translates this information to provide the differentiating conducive environment and proper signal at the right time to ensure successful completion of the differentiation process (Zakrzewski et al. 2019). Briefly, during embryonic organogenesis of the heart, a primitive streak is developed from the mesoderm, the intercalating region between the ectoderm and endoderm. The formation of primitive streak originates at the gastrulation phase of vertebral embryonic development due to the migration of anterior mesodermal cells. The primary streak then contributes to the heart forming cardiac progenitor cells, located at the mid-streak position of the primitive streak (Camp et al. 2012). Upon the morphogen signals from adjacent cells, the cardiac mesoderm is induced, a crucial step in developing the heart. The morphogens involved in cardiomyogenesis include three families of growth factors that regulate

the terminal cardiomyocyte differentiation in vivo and include BMPs belonging to the TGF-β superfamily, Wingless/INT proteins (Wnts), and FGFs (Parikh et al. 2015). The sequential expression of these morphogens is very crucial for stage-specific differentiation during cardiomyogenesis. The schematic representation of stages specific to cardiomyogenesis lineage differentiation along with their profiled markers is illustrated in Fig. 1.

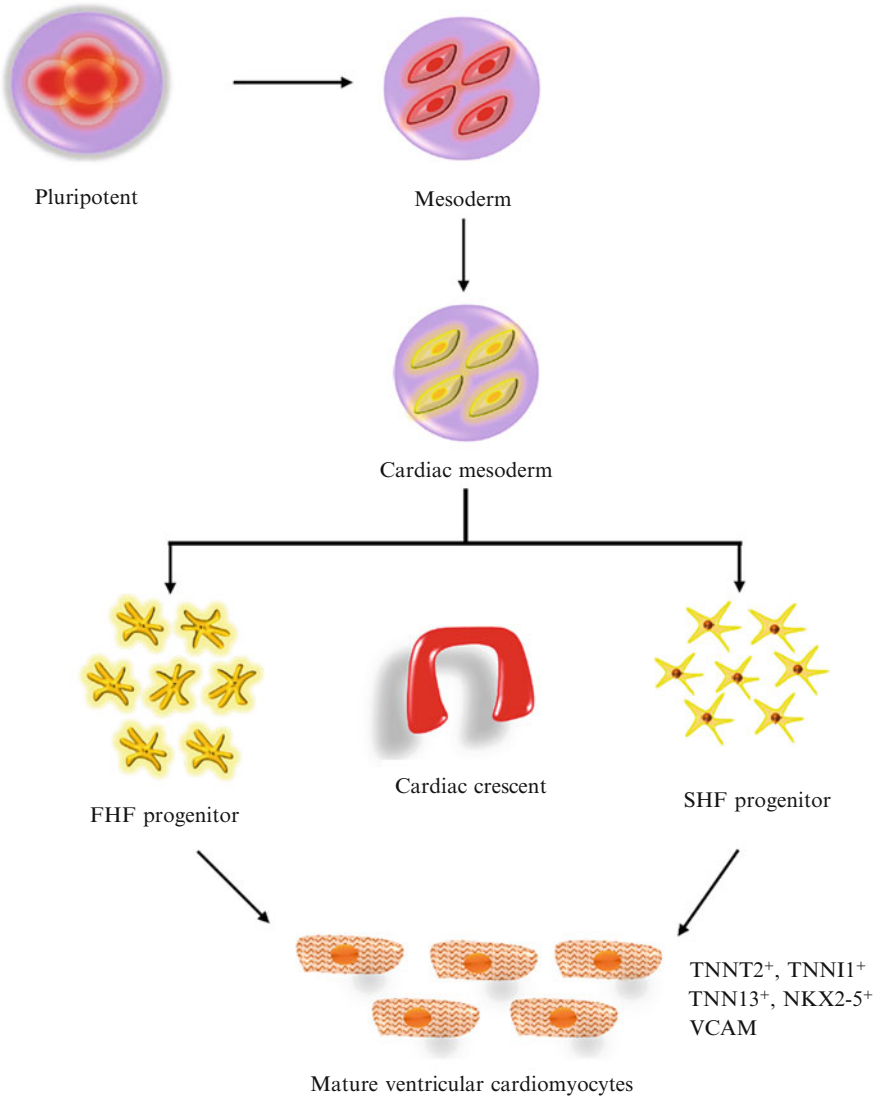

Fig. 1 Schematic representation of major cardiac specific markers at various stages of differentiation

Bone Morphogenetic Proteins (BMPs)

The site of myocardial origin is the posterior epiblast which was formed before primitive streak formation. The myocardial primitive cells present in abundance at the anterior part of the primitive streak migrate further to the anterior lateral to form a lateral mesodermal plate (Marvin et al. 2001; Sweetman et al. 2008). Furthermore, the mesoderm tissue bifurcates into somatic and splanchnic layers in which cells are falling inside the splanchnic mesoderm to form a cardiac crescent or the primary heart field. Further, the primary heart field cells successively form the myocardium and endocardium. The cells falling beyond the primitive streak are destined to ventricular myocytes and atrial myocytes, thus forming a mature heart (Eisenberg et al. 2004). The BMP family of proteins plays a vital role in almost all stages from cardioblast formation to later stages of heart organogenesis (van Wijk et al. 2007). BMPs are involved in a vast sequence of biological processes, such as perpetuation, lineage commitment and differentiation, migration, and apoptosis.

Six BMPs, i.e., BMPs 2, 4, 5, 6, 7, and 10, are expressed in various regions of the heart and govern the heart architecture and homeostasis (Goumans et al. 2018). BMPs play a context-dependent role as both agonist and antagonist at a state-specific lineage differentiation process. An accurate interplay of BMP stimulation and inhibition is essential for stage-specific differentiation of pluripotent stem cells into cardiomyocyte-like cells (Liu et al. 2004; Prall et al. 2007; Ying et al. 2003). This strategy is mimicked in vitro to manipulate the lineage induction studies from stem cells in vitro. The components of the BMP signaling pathway trigger mesoderm specification from the primitive streak and facilitate cardiac specification during vertebrate heart organogenesis. It contributes to a plethora of regulatory processes in myocardio-vasculogenesis. BMP is necessary for the induction of second heart field-derived heart tube genesis from the distal borders of the mesoderm. Additionally, BMPs are also involved in forming septovalvulogenesis in the atrioventricular canal and chambers of the ventricles. BMP signaling requires type I and II heterodimeric receptors, which, upon binding of BMPs, further phosphorylate the downstream receptor-regulated R-Smads (Smad1, Smad5, and Smad8) (Derynck and Zhang 2003). Upon phosphorylation, R-Smad and Smad4 form a complex, which is translocated into the nucleus to initiate gene expression.

Wang et al. have shown that transient treatment with BMP-2 prompts c-kit$^+$ BM-MSCs to differentiate into functional CMs (Wang et al. 2018). Further transplantation of BMP-2-induced c-kit$^+$ MSCs promotes the repair of the infarcted myocardium and improves cardiac function. However, the data showed poor survival of engrafted stem cells in the ischemic and inflammatory microenvironment. Therefore, the strategy addressing the long-term survival of transplanted cells is important for successful therapy.

Wnt Pathway

Wnt signaling pathway significantly contributes to tissue architecture, lineage commitment, differentiation, cell migration, cell polarity, proliferation, etc., during cardiogenesis (Ring et al. 2014). Hence, inappropriate regulation of Wnt signaling

may lead to a malformed heart and delay the process of repair in the infarcted heart. Wnt signaling consists of three major pathways. All types are activated by the binding of the Wnt protein ligand to a Frizzled family receptor, and further, the signal will be relayed by Dishevelled (Dsh) protein intracellularly (Komiya and Habas 2008). The canonical Wnt/β-catenin pathway, non-canonical Wnt/planar cell polarity (PCP) pathway, and Wnt/Ca^{2+}-dependent pathway are the three major types of pathways committed for the aforesaid function. Mouse ESC differentiation studies have revealed that Wnt/β-catenin signaling exerts stage-specific, context-dependent role in cardiovascular developmental biology (Paige et al. 2010). Early-phase activation of the Wnt/β-catenin pathway in embryoid bodies (EBs) facilitates ESC differentiation into cardiomyocyte lineage while suppressing their differentiation into hematopoietic and vascular cell lineages (Rungarunlert et al. 2009). On the other hand, late-phase activation of Wnt/β-catenin signaling in EBs blocks myocyte lineage and augments the expression of hematopoietic/vascular-specific markers via the downregulation of BMP signaling (Noseda et al. 2011; Zhang et al. 2016). Thus, Wnt/β-catenin signaling unveils biphasic but antagonistic effects on cardiovascular development, highly dependent on the developmental stage (Naito et al. 2006). Therefore, it is now well-understood that the canonical Wnt/β-catenin signaling pathway is pro-cardiogenic in the early pre-cardiac mesoderm phase but inhibitory during the terminal phase of cardiac differentiation. Therefore, given the significance of Wnt/β-catenin signaling in stimulating stem/progenitor cells, it is crucial to define its regulation to produce adequate derivatives of cardiac and vascular progenitors for future therapeutic use.

The non-canonical or β-catenin-independent Wnt signaling also plays a significant role in developing second heart field (SHF) cells. Wnt5a and Wnt11 are the two non-canonical Wnt ligands that stimulate cardiac differentiation in ESCs and adult stem cells and counterbalance the β-catenin-dependent SHF proliferation in the outflow tract. Moreover, there is strong evidence that Wnt5a and Wnt11 act non-canonically in the SHF (Cohen et al. 2012; Buikema et al. 2013). Another study showed C-jun N-terminal kinase (JNK) and protein kinase C (PKC) act downstream of β-catenin-independent Wnt signal that was mediated via Wnt 11 ligand (Wang et al. 2020). Furthermore, inhibiting the role of either JNK or PKC signaling blocked the ability of Wnt11 to induce cardiac specification. On the other hand, co-activating JNK and PKC persuade cardiac specification, proposing both the RhoA/JNK and Ca^{2+}/PKC pathways arbitrate Wnt11 signaling. In summary, triggering cardiac differentiation by Wnt11 via non-canonical Wnt signaling is co-mediated by JNK and PKC signaling.

Fibroblast Growth Factor Signaling Pathway

FGF plays a prime role in organ architecture and the establishment of relative proportions of compartmentalization of organs. FGF8 mutant zebrafish heart showed reduced organ size and proportionality (Marques et al. 2008). During cardiac lineage commitment, loss of FGF signaling impedes the establishment of ventricular and atrial CMs, with a more decisive influence on ventricular cells. Thus, it is now well-

established that FGF signaling initially governs the heart volume and proportion, and during the latter phase, it improves the ventricular section by amendable cell number and beginning of differentiation (Itoh et al. 2016). It is important to mention that FGF induces various functions in lineage differentiation via its paracrine mechanism. Among the various FGFs, FGF8, FGF9, FGF10, and FGF16 function as paracrine signals during embryonic heart development. Other FGFs such as bFGF, FGF9, FGF10, and FGF16 also involved in Schematic representation of major cardiac specific markers at various stages of differentiation via its paracrine activity. Hence, FGF2 and FGF10 are frequently an integral part of the cardiac differentiation protocols to obtain CMs from cultured stem cells. In addition, FGF10 was also used in cardiac reprogramming protocols from cardiac fibroblast (Beenken and Mohammadi 2009).

FGF2 pedals the differentiation and deployment of resident cardiac precursors/stem cells into functionally competent CMs to facilitate the management of cardiac diseases (Rosenblatt-Velin et al. 2005). Previous studies suggested that bFGF was pro-cardiogenic and also controlled the biological course of myogenesis. bFGF showed synergistic activity with hydrocortisone in triggering the cardiac differentiation of MSCs (Hafez et al. 2016). MSCs genetically modified to overexpress bFGF showed high cell viability under hypoxic conditions (Song et al. 2005). Moreover, pretreatment of MSCs with bFGF showed enhanced engraftment and lineage differentiation potential with augmented cardiac function under in vivo MI model study.

Cardiac-Specific Transcription Factors

GATA Family of Transcription Factors

The double zinc finger cysteine-rich (CysX2-CysX17-CysX2-Cys) domain acts as a transcription factor by binding to the conserved specific region of DNA (A/T)GATA (A/G), hence the name GATA. The GATA family of transcription factors consists of six known members GATA-1–6, in which GATA-1, GATA-2, and GATA-3 modulate hematopoiesis and GATA-4, GATA-5, and GATA-6 regulate heart developmental events, such as differentiation, migration of CMs, etc. (Lentjes et al. 2016). GATA-4 is considered the first transcription factor known to be expressed in myocytes, and it plays a vital role in cardiac growth and development. Also, it is involved in various pathological conditions of the heart, such as cardiac hypertrophy and heart failure (Pikkarainen et al. 2004). GATA-4 is also known to trigger the co-transcription factors and other relevant late cardiac markers mediating myogenesis from the early to late differentiation phase (Yilbas et al. 2014). Previous in vitro and in vivo studies revealed that GATA-4 expression levels remain unaltered by hypertrophic chemical stimulants. However, mechanical and electrical stimuli have been associated with hypertrophic phenotype in neonatal rat cardiomyocyte studies (Chow et al. 2013). Additionally, the post-translational modifications of GATA-4 and its synergistic interaction with other co-factors play a prominent role in the sequential stimulation of myogenesis.

GATA-4 interacts with various downstream targets such as extracellular signal-regulated kinase-2 (ERK-2), protein kinase C (PKC), and JAK-STAT pathways, where PKC phosphorylation augments GATA-4 DNA-binding activity and STAT-1 functionality and facilitates physical interaction with GATA-4 to activate other promoters (Liang et al. 2001; Wang et al. 2005). Glycogen synthase kinase-3β (GSK-3β) downregulates GATA-4 activity via nucleo-cytoplasmic shuttling of GATA-4. Phosphorylation of the N-terminal domain of GATA-4 protein by GSK-3β leads to the downfall in the GATA-4-mediated transcriptional activity (Sugden et al. 2008). GATA-4 works with other transcription factors and co-activators, such as p300, GATA-6, MEF-2, NFAT, NKX 2.5, SRF, dHAND, and YY1 (Kohli et al. 2011). Therefore, the GATA-4 transcription factor has been well-studied for its involvement in hypertrophic cardiomyopathy and use as a novel target for treating cardiac hypertrophy.

Myocyte Enhancer Factor-2 (MEF-2) Family

The MEF-2 transcription factor contains four family members MEF-2A, MEF-2B, MEF-2C, and MEF-2D, where MEF-2A and MEF-2D are primarily involved in regulating the immune system and striated muscles. MEF-2C has a critical role in myocyte differentiation and postnatal growth of the myocardium (Pon and Marra 2016). MEF-2 downstream pathways include p38 MAPK, MAPK1, and PI3K-Akt signaling domains regulating myocyte differentiation and cardiac hypertrophy (Pon and Marra 2016). Moreover, MEF-2 is also a significant effector of Ca^{+2} signaling and activates Ca^{+2}-binding proteins, calcium calmodulins (CaMs), and their downstream modulators calmodulin kinases (CaMKs) and calcineurin, which induce cardiac hypertrophy (Kohli et al. 2011). CaMKs negatively regulate the expression of MEF-2 via phosphorylation of class II histone deacetylase (HDACs), followed by dissociation of HDAC-MEF-2 complex. Activation of MEF-2 sustained via co-activators with the HAT, such as CREB binding protein (CBP) and p300. MEF-2 also interrelates with various co-activators, i.e., GATA-4 (Morin et al. 2000), NFAT (Blaeser et al. 2000), MyoD (Berkes and Tapscott 2005), and Smad proteins (Quinn et al. 2001). This cross-talk renders MEF-2 an essential component of the hypertrophic pathway and an effector triggering the genes resulting in the manifestation of hypertrophic phenotype.

NKX-2.5

The homeobox protein Nkx-2.5 has a helix-turn-helix motif binding to the specific consensus DNA sequence T(C/T)AAGTG. Any hypertrophic stimulus upregulates Nkx-2.5 expression and its downstream target genes. Nkx-2.5 activity is modulated by interactions with other transcription factors including GATA-4, MEF-2, eHAND, and other co-activators (Morin et al. 2000; Chen and Schwartz 1995; Thattaliyath et al. 2002; Palmer et al. 2001; Brown et al. 2004). Synergistic activity of Nkx-2.5

and GATA-4 further triggers the downstream of many cardiac gene promoters like ANP. Overall, enhanced activity of NKX 2.5 is correlated with hypertrophic conditions, thus making Nkx-2.5 an attractive target for the developing therapeutic strategies targeting myocardial hypertrophy.

Serum Response Factor and Myocardin

Serum response factor (SRF) is a MADS-box transcription factor that binds its transcriptional co-activator myocardin specifically expressed in smooth and cardiac muscle cell lineages (Wang et al. 2001, 2002). Myocardin alone, by default, does not bind with DNA; instead, it forms a stable ternary complex with SRF and then brings about the transactivation of target genes. Myocardin has a critical role in developing the heart by participating as a molecular switch in controlling genes associated with smooth muscle cell proliferation and differentiation (Wang et al. 2001, 2004). SRF induces pro-cardiogenic activity in association with myocardin and coordinately activates the muscle-specific genes. Overexpression of myocardin in neonatal rat CMs was manifested as hypertrophy of the cells, upregulation of ANF, and intensely organized sarcomeres. These molecular and cellular changes strongly suggest a pivotal role of myocardin in cardiac remodeling and hypertrophy. Therefore, targeting this nuclear effector may disrupt mechanistic links and abrogate the disease manifestation.

HAND Transcription Factors

HAND family of proteins consists of the basic helix-loop-helix transcription factors involved in cardiac development and related pathologies (Oka et al. 2007). It comprises HAND1, exclusively expressed in the right ventricle, and HAND2 expressed in both ventricles and atria. Although the direct correlation of HAND genes with hypertrophy is not well-established, its downstream effector genes are also not entirely known. These transcription factors act as co-activators together with the other cardiac transcription factors and synergistically trans-activate cardiac genes. Upon binding with p300, HAND2 interacts with GATA-4 to activate ANP, BNP, and α-MHC. HAND1 also activates the ANP gene by cooperating with Nkx-2.5 (Akazawa and Komuro 2003). This transcription factor is not well-studied, and hence further studies are warranted in the future.

Two-Dimensional (2D) Differentiations of Stem Cells into Cardiomyocytes

Numerous protocols have been developed to enrich the CMs that include genetic modification (by the expression of MYH6), using surface protein as a specific marker for CMs (SIRPA (signal-regulatory protein alpha)), or selection based on

targeting highly active mitochondria using a mitochondrial membrane potential marker, tetramethylrhodamine methyl ester perchlorate (TMRM) (Tsai et al. 2019; Dubois et al. 2011; Skelton et al. 2014; Hattori et al. 2010). The in vitro culture aims to harvest a high number of CMs without extensive manipulation to avoid undesired cellular and molecular effects. The suitable, reliable protocol ensures a high percentage yield of CMs expressing cardiac-specific marker TNTN2 in both hESCs and hiPSCs without using any purification protocol. Although well-defined differentiation conditions have significantly amended the reproducibility of differentiation, inter-experimental reproducibility remains a key challenge.

Status of iPSC-Derived Cardiomyocytes

Standard protocols for the differentiation of iPSCs into CMs generally result in heterogeneous cell populations with small, misaligned, immature CMs of varied shapes and sizes, lacking the well-formed myofibrils and T-tubules, polyploidy, polarized intercalated discs, or abundant mitochondria (Karbassi et al. 2020). Ideally, the adult cardiomyocyte phenotype both gives the structural framework of the cell and directly establishes critical functional properties of the cell, such as electrophysiology and contractility (Henderson et al. 2017; Tu et al. 2018). Previous studies have demonstrated that cardiomyocyte membrane capacitance is directly proportional to cell surface area. Therefore, elongated, an anisotropic shape, resulting in long myofibrils with laterally organized sarcomeres, permits efficient cardiac contractility (Gautel and Djinović-Carugo 2016). In summary, numerous studies showed the successful differentiation of iPSCs to beating CMs under a 2D platform; however, the generated CMs exhibit a profile analogous to fetal or primitive CMs (Di Baldassarre et al. 2018). Consequently, an immature cardiomyocyte phenotype is not an appropriate model for various preclinical and clinical studies.

The changes in the structure from immature to mature CMs lead to functional variance restraining the potential of hPSC-CMs to recapitulate normal development or model human disease, thus limiting its clinical and research utility. Hence, the success of generating the physiologically competent cardiomyocyte phenotype derived from iPSCs is technically limited using the current protocols and methods of inducing differentiation. At this juncture, differentiation protocols using scaffolds have gained more attention since it renders the cultured cells in an environment which mimics their natural niche or environment. The most advanced culture technique is the generation of organoids, which are the miniature microphysiological system mimicking in vivo milieu. Although advanced organoid culture systems obtained from native decellularized extracellular matrices' (ECMs') frameworks have been developed and commercialized, their reproducibility remains a serious concern (Aisenbrey and Murph 2020). Synthetic scaffolds, such as hydrogels and their derivatives, which offer fully defined hydrogel-based scaffold recapitulating in vivo cellular microenvironment, are also used for the generation of organoids. Their availability facilitates amenable heterogeneous culture systems with long-term viability.

Current Technical Limitations

The cumbersome invasive isolation procedures followed by technical difficulties in culturing human primary CMs are the potential reasons for the deficient information about the cardiomyocyte biology. Thus, culturing of CMs from the non-human origin and experimental animal models were the only viable options for carrying out fundamental explorations in cardiac research. However, mouse and human CMs exhibit various distinct maturation features like MYH6/7 isoform switching, contraction rates, and action potential profiles (Reiser et al. 2001; Li et al. 2013; Yang et al. 2014; Nugraha et al. 2019). The animal models and non-human origin model cells are often considered inaccurate model systems to study the pathophysiological conditions of CMs in humans. Therefore, the human origin cardiomyocyte-based model system remains an important contemporary need for the understanding of cardiomyocyte biology. Thus, the human-derived CMs will provide a more relevant model system for understanding the pathophysiology of CMs, besides providing an efficient assay platform for drug discovery and drug toxicity. Furthermore, manipulating the human-derived competent cardiac organoids using the appropriate delivery system and bio-adhesive glues can be potentially employed in tissue engineering approaches for regenerating damaged myocardium.

State of the Art

Stem cells have enormous plasticity to differentiate into various desired lineages. However, the idea of using stem cells to differentiate into the cardiac lineage has so far been a relatively tough proposition for researchers around the world due to its complex nature. ESCs are the choice of cells in this regard, yet ethical concern has restricted its clinical utility. Similar to ESCs, the iPSCs also have enormous differentiation potential if provided with specific conditions and cues. Although cardiomyogenic differentiation of iPSCs has been extensively reported in the literature, maturation defects in iPSC-derived CMs remain a challenge and, hence, snag their clinical use (Ahmed et al. 2011a, b; Buccini et al. 2012; Li et al. 2013; Yang et al. 2014; Nugraha et al. 2019). Using the current standard 2D differentiation-induction protocols in vitro, iPSC-derived CMs lack the dynamic physical cues, i.e., tissue-like framework; the absence of non-cardiac source cells, i.e., endothelial cells, myofibroblasts, etc.; and the environmental cues, i.e., hemodynamic force, necessary to induce and propagate their maturation even after prolonged culture conditions (Gautel and Djinović-Carugo 2016). Therefore, a novel and technically designed 3D platform has been developed that controls the elongated anisotropic rod-shaped morphology and facilitates successful maturation of the derived CMs (Philippe et al. 2020; Yokoyama et al. 2021). Such a platform prompts morphological growth and maturation in a brief span, which is highly advantageous for its applications. Therefore, a 3D culture system is considered a more realistic model for developing a multicellular in vitro tissue construct that mimes its corresponding in vivo organ for various theranostic applications (James et al. 2021).

Regulation of Lineage Differentiation in Stem Cells

The epigenome or the chromatin landscape of stem cells determines their potency and long-term self-renewing ability. Thus, epigenetics plays a significant regulatory role in determining stem cell fate. Modern biologists define epigenetics as the study of heritable changes in gene expression that does not involve changes to the underlying DNA sequence – a change in phenotype without a change in genotype (Waddington 2012). Each cell type has a similar genome in a complex mammalian system, but the gene expression profiles vary according to the specific cell type depending on its function (Govarthanan et al. 2021). The ability of stem cells to self-renew and differentiate into various downstream lineages is highly dependent on specialized chromatin signatures that keep stem cell-specific genes active and vital differentiation factors repressed but poised for activation (Yoshida et al. 2019). Numerous but specific epigenetic changes occur concomitantly during the differentiation of MSCs to particular cell types. Thus, an epigenetic landmark not only dictates the expression profile of the stem cells; it also determines the ability of differentiation into various lineages effectively.

Differentiation Cues

Small Molecule Inhibitors

Small molecule inhibitors are biologically active moieties employed as inductive cues to modulate cell fate. Thus, small molecules are being used as versatile tools to probe the events of developmental processes besides treating cancers and degenerative diseases. Small molecules can also modify or manipulate stem cell fate by inducing their self-renewal property or enhancing their lineage differentiation potential (Ying et al. 2003; Sato et al. 2004; Chen et al. 2006; Li et al. 2011). Interestingly, these molecules play a crucial role in the reprogramming of somatic cells by replacing one or two transcription factors and thereby enhancing or accelerating the reprogramming process (Shi et al. 2008; Mikkelsen et al. 2008; Huangfu et al. 2008; Li et al. 2009; Pasha et al. 2011). Therefore, the addition and withdrawing effects of small molecule inhibitors aid us to understand the developmental and regulatory phenomenon to devise a reproducible protocol for cellular reprogramming (Chen et al. 2006; Ying et al. 2003; Sato et al. 2006; Li et al. 2009, 2011; Shi et al. 2008; Mikkelsen et al. 2008; Huangfu et al. 2008). Given their ease of use, they are employed in various reprogramming protocols, as illustrated in Fig. 2.

Role of Small Molecule Inhibitors in Stem Cell Biology

Advanced reprogramming protocols are being developed based on non-integrating and non-genetic delivery methods in an attempt to establish commercial as well as

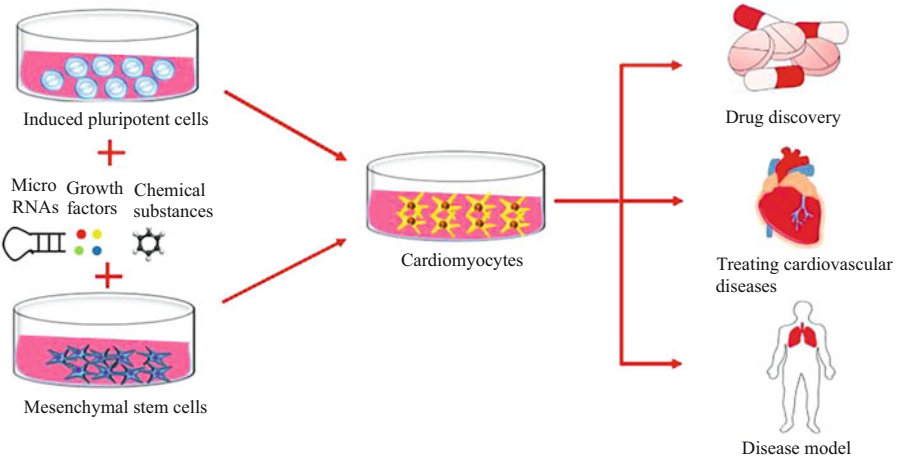

Fig. 2 Schematic representation of cues employed for differentiation of stems cells and its application

clinical-grade iPSC preparations (Yu et al. 2009; Zhou et al. 2009; Kim et al. 2009; Yakubov et al. 2010; Huangfu et al. 2011; Wang et al. 2018). Although it requires the repeated addition of small molecules, it is simple and cost-effective. A chemical-based reprogramming approach has also been reported to replace one or two transcription factors to generate iPSCs (Pasha et al. 2011; Hou et al. 2013). Chemical modulators are categorized as epigenetic modifiers, modulators of cell signaling pathways, cell death and stress alleviators, and modulators of metabolism, thus interacting with the proteins by either activating or inhibiting their activity to control the fate of the cells (Cao et al. 2016). Some of the epigenetic modifiers are solely used to increase the yield of iPSCs. Some of the typical examples based on the mode of activity of these modifiers are BIX01294, a diazepine-quinazolinamine derivative and histone-lysine methyltransferase (HMTase) inhibitor that modulates the epigenetic status of chromatin (Shi et al. 2008), and HDAC inhibitors like valproic acid (VPA), trichostatin A (TSA), and suberanilohydroxamic acid (SAHA) that block HDAC resulting in the hypoacetylation of histones and an increase in gene expression in the target cells, which can decrease transcription (Mikkelsen et al. 2008).

Our group has deciphered the role of 5-azacytidine (5-Az) and CHIR99021 in augmenting the differentiation of MSCs. We found that inherently hypomethylated cardiac-enriched gene promoter regions in MSCs might render 5-Az-induced protocols redundant (Govarthanan et al. 2020a, b). Wnt agonist CHIR99021 primed MSCs showed a state of increased potency (Govarthanan et al. 2020a, b). Thus, manipulation of MSCs using potent pharmacological cues enhanced their transdifferentiation potential and augmented their clinical utility. DNA methyltransferase inhibitor RG108, a non-nucleoside analog, acts by direct binding to the methyltransferase enzyme, blocking the enzyme active site (Pasha et al. 2011; Cheng et al. 2015). BIX01294 also inhibits histone methyltransferase enzyme

G9a, and therefore, it is used for replacing Sox2 and c-Myc in generating iPSCs (Takahashi et al. 2007; Li et al. 2009; Silva et al. 2008).

Somatic cell reprogramming efficiency is improved in humans and mice using VPA and DNA methyltransferase inhibitor (Li et al. 2009). Also, modulators of signaling pathways, such as MEK inhibitor (PD0325901), cell-permeable inhibitor of the MEK/ERK pathway that inhibits the activation and downstream signaling of MEK (Takahashi et al. 2007), and agonist of Wnt pathway, GSK3b inhibitor (CHIR09921), were used for the complete and stable establishment of pluripotency in neuronal stem cells (NSCs) using two factors Oct4 and Klf4 (Li et al. 2009). Cheng et al. have reported chemically induced NSCs (ciNSCs) from mouse tail-tip fibroblasts by using a cocktail of VPA (HDAC inhibitor, HDACi), CHIR99021 (GSK3β inhibitor), and RepSox (a cell-permeable, selective inhibitor of the TGF-β type I receptor) under physiological hypoxic conditions (5%O_2) (Cheng et al. 2015). Another study showed endogenous Oct4 activation using Wnt pathway agonists (NaB, LiCl) and SB431542 (a selective and potent inhibitor of the TGF-β/Activin/NODAL pathway that inhibits activating-like kinases (Alk5, Alk4, and Alk7) by competing for the ATP binding site) in mouse embryonic fibroblasts under physiological hypoxic conditions and obtained ciNSCs that resembled NSCs in gene expression profile and differentiation potential (Cheng et al. 2014). Pei et al. also demonstrated the conversion of human fibroblasts into neurons using a cocktail of small molecules (Pei et al. 2015). Table 1 enlists the broad activity of small molecule inhibitors in reprogramming on the various cellular sources.

Microenvironment

Extracellular matrix (ECM) is a dynamic and complex milieu in the stem cell niche governing the cell behavior and significantly influences stem cell fate toward cardiac differentiation (Gong et al. 2021). In vitro studies have revealed that stem cell culturing in biomaterial systems induces cardiac-specific gene expression and cardiomyogenic differentiation potential (Lin et al. 2016). For example, BM-derived MSCs cultured on collagen nanoparticles display upregulated cardiac-specific gene expression compared to MSCs cultured without collagen nanoparticles. On the other hand, MSCs cultured on graphene increase their cardiac-specific gene expression (Park et al. 2014). Compared to collagen, graphene is found to have conductive properties; however, issues related to biocompatibility need to be addressed before proceeding to applications. Substrates of graphene and collagen that can be used as scaffolds for achieving cardiac differentiation of MSCs by mimicking the ECM properties associated with the native cardiac microenvironment are currently under research (Rashedi et al. 2017). MSCs stimulated under electrical impetus show early cardiac transcription factors, such as GATA4 and Nkx2.5.

Therefore, electroconductive influence is considered as a significant parameter to achieve cardiac differentiation in MSCs (Rashedi et al. 2017). Adipose-derived MSCs treated with HDAC inhibitor TSA cultured on the fibrin scaffold showed activated gene transcription profiles of CMs (Song et al. 2011). Polyurethane nanofibers induced

Table 1 Summary of reported small molecule inhibitors and its pleiotropic effects

Compound name	Identity	Function	Source
Valproic acid (VPA)	HDAC inhibitor	Promotes MEF reprogramming efficiency and enables Oct4- and Sox2-mediated reprogramming of human fibroblasts Facilitates protein-mediated reprogramming of MEFs	Mouse embryonic fibroblast and human fibroblasts Mice adult pancreatic exocrine cells
Suberoylanilide hydroxamic acid	HDAC inhibitor	Promotes MEF reprogramming efficiency	Mouse embryonic fibroblast
Trichostatin A	HDAC inhibitor	Promotes MEF reprogramming efficiency	Mouse embryonic fibroblast
Sodium butyrate (NaB)	HDAC inhibitor	Enhances reprogramming efficiency of human adult or fetal fibroblasts Facilitates Oct4-only-mediated reprogramming when combined with A-83-01/PD0325901/PS48	Human adult or fetal fibroblasts Human keratinocytes
BIX01294	G9a HMT inhibitor	Enables NPC reprogramming mediated by Oct4 and Klf4 or substitutes for Oct4 in NPC reprogramming Promotes MEF reprogramming mediated by *Oct4* and *Klf4*	Mouse ESCs and fetal brain cells Mouse embryonic fibroblast
RG108	DNMT inhibitor	Promotes MEF reprogramming mediated by Oct4 when combined with BIX01294	Mouse embryonic fibroblast
5-Azacytidine	DNMT inhibitor	Increases MEF reprogramming efficiency	Mouse embryonic fibroblast
Parnate	LSD1 inhibitor	Enables reprogramming of human keratinocytes mediated by Oct4 and Klf4 Facilitates the conversion of mEpiSCs to native pluripotent state	Human neonatal epidermal keratinocytes Mouse epiblast stem cells
PD0325901	MEK inhibitor	Blocks the differentiation pathway of ESCs and supports self-renewal Supports ESC derivation from refractory strains or species Facilitates conversion of mEpiSCs and hESCs to native pluripotent state Facilitates generation and maintenance of mESC-like rat or human iPSCs Facilitates rapid and efficient generation of fully reprogrammed hiPSCs Enables Oct4-mediated reprogramming when combined with A-83-01/NaB/PS48	Mouse ESCs, human ESCs Mouse ESCs, rat ESCs Mouse epiblast stem cells, human ESCs Rat WB-F344 cells, human fibroblasts IMR90 Human primary fibroblasts (CRL2097 or BJ) Human keratinocytes

(continued)

Table 1 (continued)

Compound name	Identity	Function	Source
CHIR99021	GSK3 inhibitor	Supports ESC self-renewal and facilitates ESC derivation from refractory stains or species Captures and maintains lineage-specific stem cells like pNSCs; facilitates the conversion of mEpiSCs and hESCs to native pluripotency Enables Oct4- and Klf4-mediated reprogramming of MEFs or human primary keratinocytes with Parnate Facilitates generation and maintenance of mESC-like rat or hiPSCs Facilitates the neural conversion of human fibroblasts mediated by Ascl1 and Ngn2	hESCs, hESCs mESCs, rat ESCs, hESCs Human keratinocytes, hESCs, human fibroblasts IMR90 Rat WB-F344 cells Human postnatal fibroblast lines
6-Bromoindirubin-39-oxime (BIO)	GSK3 inhibitor	Promotes self-renewal of ESCs and Isl+ cardiovascular progenitors	HESCs and mESCs mESCs and human heart Isl1-positive cells
Kenpaullone	GSK3 and CDK inhibitor	Replaces Klf4 in MEF reprogramming	Mouse embryonic fibroblast
PD173074	FGF receptor inhibitor	Supports mESCs' self-renewal Facilitates the conversion of mEpiSCs to native pluripotent state	mESCs Mouse epiblast stem cells
SU5402	FGF receptor inhibitor	Supports mESCs' self-renewal	mESCs
A-83-01	ALK4, ALK5, ALK7 inhibitor	Facilitates the conversion of mEpiSCs to native pluripotent state Enables generation and long-term maintenance of mESC-like human iPSCs Enables Oct4-mediated reprogramming when combined with PD0325901/NaB/PS48	Mouse epiblast stem cells Human primary fibroblasts (CRL2097 or BJ) Human keratinocytes
SB431542	ALK4, ALK5, ALK7 inhibitor	Captures and maintains pNSCs when combined with CHIR99021 Facilitates rapid and efficient generation of fully reprogrammed hiPSCs Facilitates the neural conversion of human fibroblasts mediated by Ascl1 and Ngn2	hESCs Human primary fibroblasts (CRL2097 or BJ) Human postnatal fibroblast lines

(continued)

Table 1 (continued)

Compound name	Identity	Function	Source
E-616452	ALK4, ALK5, and ALK7 inhibitor	Replaces Sox2 in MEF reprogramming	Mouse embryonic fibroblast
LDN193189	ALK2, ALK3, and ALK6 inhibitor	Facilitates the neural conversion of human fibroblasts mediated by Ascl1 and Ngn2	Human postnatal fibroblast lines
Compound E	c-secretase inhibitor	Accelerates the generation of pNSCs	hESCs
JAK inhibitor I	JAK inhibitor	Inhibits the generation of iPSCs in iPSC-TF-based transdifferentiation	Mouse fibroblast cells
Pluripotin (SC1)	Ras GAP and ERK inhibitor	Maintains mESC self-renewal	mESCs
Y-27632	ROCK inhibitor	Improves survival of hESCs upon dissociation	hESCs
Thiazovivin (Tzv)	ROCK inhibitor	Improves survival of hESCs upon dissociation Facilitates rapid and efficient generation of fully reprogrammed hiPSCs	hESCs Human primary fibroblasts (CRL2097 or BJ)
StemRegenin1	AhR antagonist	Enables ex vivo expansion of CD34+ HSCs	Human HSCs
PS48	PDK1 activator	Enables OCT4-mediated reprogramming with A-83-01, NaB, and PD0325901	Human keratinocytes
BayK8644	L-type Ca^{2+} channel agonist	Promotes MEF reprogramming mediated by Oct4 and Klf4 when combined with BIX01294	Mouse embryonic fibroblast
Forskolin	PKA agonist	Induces Klf4 and Klf2 expression to facilitate hESCs' conversion into a native pluripotent state	hESCs

Modified and adapted from Zhang et al. (2012)
HDAC histone deacetylase, *HMT* histone methyltransferase, *DNMT* DNA methyltransferase, *LSD1* lysine-specific demethylase 1, *MEK* MAPK kinase, *GSK3* glycogen synthase kinase 3, *FGF* fibroblast growth factor; *ALK* activin A receptor-like kinase, ALK2, ACVR1; ALK3, BMPR1A; ALK4, ACVR1B; ALK5, TGFBBR1; ALK6, BMPR1B; ALK7, ACVR1C, *JAK* Janus kinase, *RasGAP* Ras GTPase-activating protein, *ERKs* extracellular signal-regulated kinases, *PDK1* phosphoinositide-dependent kinase 1, *ROCK* Rho-associated coiled-coil-containing protein kinase, *AhR* aryl hydrocarbon receptor, *PKA*, protein kinase A, *MEF* mouse embryonic fibroblasts, *ESC* embryonic stem cell, *mESC* mouse ESC, *hESC* human ESC, *mEpiSC* mouse epiblast stem cell, *iPSC* induced pluripotent stem cell, *NPC* neural progenitor cell, *pNSC* primitive neural stem cell, *HSC* hematopoietic stem cell

cardiac gene expression under 3D conditions; especially, it rendered MSCs to attain aligned morphology compared to the 2D cultured conditions. Therefore, scaffolds involving experimental conditions strongly favor cardiomyogenesis in vitro.

Exosomes for In Vitro and In Vivo Cardiac Applications

3MSCs secrete soluble and insoluble factors, including growth factors, cytokines, exosomes, etc., as a part of their paracrine activity capable of regenerating the damaged cardiac tissue. The paracrine action of MSCs is now an established mechanism by which the transplanted cells contribute by supporting the intrinsic repair mechanisms (Haider and Aziz 2017). Moreover, it has also led to the emergence of a cell-free therapy approach for myocardial repair (Haider and Aslam 2018). The transplanted MSCs activate the endogenous stem cell its home to the infarcted myocardium, participate in the repair process, as well as maintain the regional tissue homeostasis (Lei and Haider 2017). MSCs also secrete exosomes containing their specific cargo, which is used for intracellular trafficking and communication, thereby modulating the fate of the endogenous stem cells' population (Zhang et al. 2015; Tsiapalis and O'Driscoll 2020; Ramesh et al. 2021). Exosomes are typically 30–100-nm-diameter released particles containing bioactive molecules like proteins, lipids, RNAs (mRNA, rRNAs, tRNAs, lncRNAs, miRNAs, and mtDNA), etc. (Li et al. 2018; Sebastião et al. 2019). Given their payload of biologically active molecules, the use of exosomes is fast emerging as a cell-free system offering exciting prospects for myocardial regenerative therapies. The secreted exosomes mainly exert anti-inflammatory, anti-apoptosis, enduring immune-tolerance, pro-angiogenic, and supporting tissue regeneration via the activation of resident progenitor cells (Haider and Aramini 2020). Current research is focused on exosomes' characterization for cell-free therapy. In this regard, exosomes derived from stem cells of diverse tissue origins and cultured under different conditions are being profiled for their heterogeneity. MSCs in native state secrete pro-angiogenic factors, while pretreatment with hypoxia or hypoxia mimicking agents promoted the release of pro-inflammatory and pro-angiogenic factors (Zhao et al. 2015; Zhu et al. 2018). Similarly, interleukin-1 primed MSCs cultured under 3D conditions mimicked tissue damage and demonstrated to release paracrine factors, such as VEGF, GCSF, and IL-1 receptor antagonist (IL-1Ra) (Frith et al. 2010; Bartosh et al. 2010; Redondo-Castro et al. 2018). Further research on native and engineered exosomes may offer a novel alternative mode of treating CVDs.

Detailed Methodology

The methods of maturation state analysis of the cells cultured under 3D cardiac organoid conditions include morphological, functional, metabolic, and gene expression profiles specific for the maturation phase.

Functional Maturation Analysis

Morphological Analysis

It includes microscopic evaluation of sarcomere alignment, well-developed sarcoplasmic reticulum, and mitochondrial size and complexity in the developed 3D

organoid model. Exhibition of elongated rod-shaped structure with the polyploidy (presence of bi- or multi-nucleus) is also counted as a parameter for determining the morphofunctional maturation of CMs.

Functional Analysis

This includes evaluating cardiac electrophysiology by patch clamping assay and providing a direct measurement of the cardiomyocyte action potential (AP). This is considered the gold standard for studying the voltage-gated channel activity, the membrane potential in resting state, polarized, and depolarized states. Analyzing the Ikl current corresponds to the mature state in CMs (Zhao et al. 2018). Another analysis is to study the calcium-induced release and fast excitation-contraction coupling events using fluo-4 fluorescent Ca^{2+} indicator (molecular probes) in the presence of pluronic F-127 (molecular probes) at a dilution of 2:1 to allow the recording of intracellular Ca^{2+} transients (Berlinguer-Palmini et al. 2014).

Metabolic Analysis

It includes the evaluation of size and complexity of mitochondria and their alignment near the sarcomere through high-resolution microscopic examination. Mitochondrial volume increases, leading to increased oxidative capacity in CMs; thereby, the mature CMs metabolically switch from glucose to fatty acid metabolism, i.e., from glycolysis to β-oxidation of fatty acids (Dai et al. 2017). As a result, more acidified ECM is generated. Further estimation of oxygen consumption rate and extracellular acidification rate would give information about CMs' metabolic maturation.

Cardiomyocyte Maturation-Specific Gene Expression Analysis

This includes monitoring the gene expression switching from MYH6 to MYH7, TNNI1 to TNNI3, TTN-N2BA to TTN-N2B, and MYL7 to MYL2 to determine the successive manifestation of the maturation phase in CMs (Opitz et al. 2004). Moreover, activation and oxidative phosphorylation of genes involved in mitochondrial biogenesis, such as PPARGC1A, PPARA, and ESRRA, and increased expression of Ca^{+2}-handling genes, such as Cav1.2, RYR2, and SERCA2, will be substantiated with the maturation events in the newly developed CMs (Guan et al. 2020; Guo and Pu 2020). Overall representation of fully mature competent CMs is illustrated in Fig. 3.

Limitations of 2D Culture

Lack of accuracy in 2D-mediated prevailing assays evaluating the cardiotoxicity of drugs currently leads to commercial withdrawal of numerous anti-cancer drugs. In addition, divergence in mouse and human physiology shows that experimental animals provide inaccurate model systems to study the pathophysiological conditions in humans (Reiser et al. 2001; Li et al. 2013; Yang et al. 2014; Nugraha et al. 2019). 2D culture-derived CMs fail to mimic the profile of adult CMs, and hence,

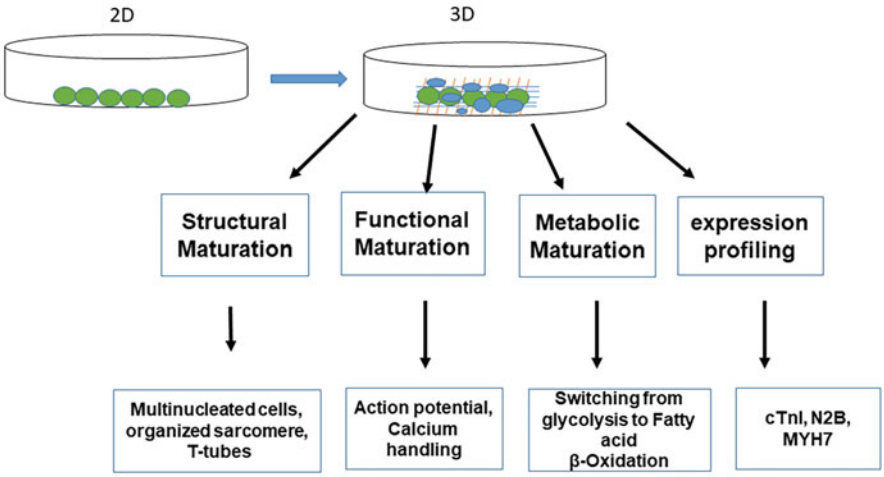

Fig. 3 Flowchart representation of mature cardiomyocyte characterization

its application in clinics may not fetch desired results. Consequently, a system needs to be devised to precisely serve the needs for in vitro and in vivo applications via a 3D advanced organoid technology to mimic stem cells' natural habitat. These bioengineered cardiac organoids – a microphysiological system, capable of re-creating certain aspects of the CMs physiology and manifested to offer multiple benefits in vitro a model system for drug toxicity screening and further developing the micropatterned cardiac organoids loaded hydrogels as a patch for effective delivery and engraftment in the infarcted myocardium for heart tissue regeneration. Figure 4 portrays the importance of a 3D culture system aimed to achieve morphological features for competent cardiomyocyte generation.

3D Cardiac Organoids

The emergent growth on the 3D cell culture market (expected to reach $1.69 billion in 2024) sets the trends for the development of new platforms enabling the re-creation of the cell's natural environment (*Source: https://www.prnewswire.com/news-releases/3d-cell-culture-market-size-worth-1-69-billion-by-2024-cagr-14-8-grand-view-research-inc–875064289.html*). 3D cell cultures provide a better resemblance to in vivo models of the morphology of organs or cells, thereby providing an efficient way in discovering novel druggable targets.

The availability of iPSC-derived CMs, together with the availability of a 3D culture system, has given impetus to human cardiomyocyte-based research to address the current challenges. It is analogous to the immature fetal cardiomyocyte-phenotype and genotype, heterogeneous in shape with a different action potential, etc. (Karbassi et al. 2020; Henderson et al. 2017; Tu et al. 2018;

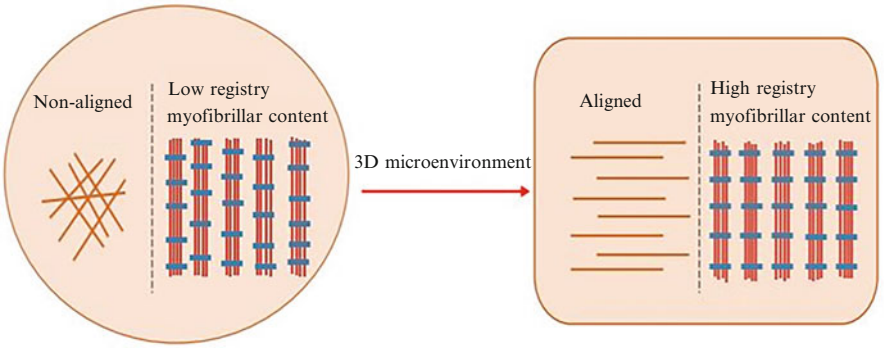

Fig. 4 Representation of current technical limitations of 2D-induced CMs and significant manipulations to achieve under 3D culture conditions to generate morphofunctionally competent CMs

Nasri et al. 2018). The internal microarchitecture is also different in terms of myosin, troponin, and sarcomeric protein contents, thus leading to dissimilar hierarchical levels of differentiation. A vast number of homogeneous populations of cells (and that too in reproducible manner) are generally required for cell-based therapy, which is difficult to achieve from the current culture conditions and protocols. iPSCs in this regard provide a renewable source of cells but with immature phenotype, as described earlier. Hence, several novel methods and culture techniques have been employed to render the highest degree of maturation in iPSC-derived CMs. Methods include identifying a synergistic cocktail of cues triggering complete differentiation profile and advanced culture technologies, including supporting native, synthetic, or hybrid biomaterial-derived scaffolds, subjecting electrical and mechanical cues, etc.

Working on the 3D Models

The choice of using patient-derived disease-specific cell lines with phenotypes and mutations in a human genetic context might open up new possibilities in precision medicine and gene therapy. The hiPSC-derived CMs with a disease-specific genotype offer in vivo tissue-like features and identify the novel leads which the 2D culture system fails to reveal. A recent study has shown the contractile deficit truncated in hiPSC-derived CMs with truncation in the sarcomeric protein Titin that was not apparent in 2D cultured CMs, but was evident in engineering heart tissue working under PDMS or silicone pillar system (Hinson et al. 2015). The various methods that enable differentiated CMs exhibiting adult cardiomyocyte characteristics, at the cellular and molecular levels, are illustrated in Fig. 5. All the aforementioned methodologies synergistically facilitate the cardiac maturation process and generate morphofunctionally competent CMs. In this regard, the 3D culture system has prompted the exploration of cardiac spheroid system to investigate therapy-induced changes in the physiology of the heart concerning contractility

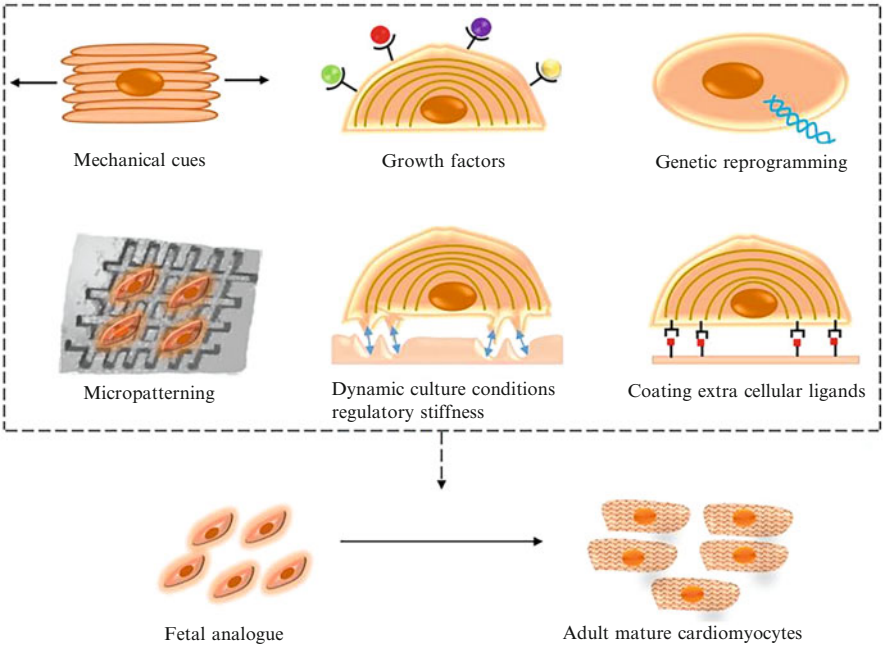

Fig. 5 3D model innovative methodologies proposed to conduct research for deriving competent morphofunctionally competent CMs for theranostic applications

and calcium handling. With these advanced technologies, few cardiomyopathy models have been demonstrated under 3D conditions to better understand the disease pathophysiology and related drug discovery.

The multicellular aggregates are the most rudimentary type of 3D culture, constructed by the self-assembly of floating cells on low-attachment surfaces via the liquid overlay method (Edmondson et al. 2014). Such suspension cultures can be maintained economically by using a sterile dish with a thin agarose film. Fetal or newborn animal-derived early CMs have already demonstrated their spontaneous tendency to form multicellular aggregate.

Many cell culture suppliers provide multi-well plates with U-shaped bottom and different undercoats for ultra-low attachment, resulting in the mass production of self-assembled spheroids or similar formats.

A soft silicone pattern can also be used to make agarose casts with several small wells for micro-tissue processing. The hanging drop method could effectively harvest uniform micro-tissues, and recently, advanced sophisticated systems incorporating hanging drops in microfluidic systems, such as perfusion and sensors, have been developed (Velasco et al. 2020). Overall, one benefit of the spheroid as a 3D cell culture is the ability to use semi-automatic methods to manufacture spheroids by filling multi-well plates with a pipetting robot for media change, drug treatment, and sample analysis in a versatile high-content screening manner.

Scaffolds Employed

The rate-limiting step in the success of the 3D culture system is the appropriate selection and usage of scaffold or assisting mold. Initially, native ECM components, such as fibrin/thrombin and/or collagen and Matrigel components, were used as a scaffold. However, the system remains undefined and, hence, leads to reproducibility concerns. Generation of bio-scaffolds using semi-synthetic and hybrid hydrogel-based systems have also been successfully designed and used for primary newborn rodent cardiomyocyte or stem cell-derived cardiomyocyte culturing as either a single-culture or co-culture system (Karbassi et al. 2020).

Similarly, 3D culture systems may also be defined by different geometries governing the shape of the organoids (Chaicharoenaudomrung et al. 2020). They are relatively more reliable in recapitulating the physiology of the CMs and further analysis, interpretation, and validation of the results on a real-time basis using these versatile 3D cardiac models. Combining with engineering technology, the microfluidic system has gained significant attention since it almost replaces the need for the animal model. The most recent innovations in this field are an organ-on-chip platform, which has been designed to incorporate micro-vasculatures using various techniques such as molding technologies, bioprinting, and combinations of these technologies. This is then used to model drug development to understand cardiovascular diseases (Ribas et al. 2016; Paloschi et al. 2021). For a long time, the problem of oxygen supply in larger artificial tissues has been a focus of tissue engineering study. Still, these processes are essentially multi-factorial, involving fine-tuned expression and post-translational processing of growth factors, relaying extracellular signals, and participation of other cell types to create a well-perfused vascular network in vitro. However, this has proven to be more difficult than expected. The modern advanced single-cell micropatterning technique involves soft lithography and micro-contact printing methods to facilitate the microfabrication of hydrogel scaffolds (Kit-Anan et al. 2021). However, these methods are labor-intensive and time-consuming. Alternatively, stencil-based single-cell patterning has been reported to generate micropatterned CMs derived from single hiPSCs. These CMs exhibited distinctive cell anisotropic morphology with an aspect ratio ranging from 1:1 square to 7:1 analogous to adult CMs with a rectangular shape (Lee et al. 2020). Therefore, future research is expected to result in patterned single-cell lithography-free, precisely robust, expedient, economical, and accessible for tissue engineering approaches.

Analytical Methods

All 3D culture methods, where the cells are available, and traditional lab techniques such as immunocytochemistry, immunohistochemistry, and cell viability/cytotoxicity assays are feasible for end-point analysis. Cell physiology methods for studying cardiac features in living tissues, such as contractions, force, calcium cycling, or electric signals, necessitate sophisticated instrumentation that varies in sensitivity

and temporal and spatial resolution. All these methods require skilled labor for obtaining work reproducibility, and it is also time-consuming. Advanced optical methods, mainly video-based analysis, for reading the stretches of sarcomeres in beating CMs, have been introduced as commercially available hardware and software packages (IonOptix, Sony) or by employing compatible open-access platform accompanying microscopes and cameras (Zuppinger 2019). In vivo imaging platform has emerged as a convenient method using white light as optical reader. Its primary advantage is that it can be repeated under a controlled culture environment. Researchers have used conventional spectroscopic methods, i.e., patch clamping and sampling with sharp microelectrode pipettes, to test electrical signals and contractile activity in cardiac 3D models. In addition to the traditional electrophysiology approaches, advanced techniques, such as multi-electrode arrays and impedance spectroscopy, are currently gaining serious consideration.

Conclusions and Outlook

Developing a prototypical system that meticulously and exactly mimics the in vivo microarchitecture will be highly beneficial for understanding cardiomyocyte biology. Numerous in vitro studies have shown that a combination of biological and chemical cues may enable an abundant supply of CMs rather than that of the single inducers alone. Nevertheless, those studies botched to reciprocate the morphofunctional characteristics of the adult CMs. Addressing the current technical limitations of conventional 2D culture systems, the modern 3D culture systems provide appropriate bio-mimetic multicellular tissue constructs for various theranostic applications. The potential modern 3D culture system is primarily intended to offer advanced replicas of culture environments that are physiologically appropriate for electrical conductivity, mechanical strength, physicochemical properties, etc. However, in-depth studies are mandatory to ensure the accuracy, validity, and reproducibility of these models, besides enhanced versatility and robustness. Thus, generating the mature engineered heart tissue demands micro- or nano-fabricator platforms using bio-ink, which requires biomaterial precisely matching in vivo cardiac ECM. Identifying novel biomaterials that can expedite specific cell-to-cell interactions is dire to guide efficient cardiac differentiation in 3D culture conditions. In a nutshell, the perfect combination of cell biology and tissue engineering with the appropriate biophysical platforms should effectively deliver functionally competent CMs for various research, clinical, and industrial purposes.

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Stem Cell Applications in Cardiac Tissue Regeneration

25

Vineeta Sharma, Sanat Kumar Dash, Piyush Kumar Gupta, Binita E. Zipporah, Khawaja Husnain Haider, and Kavitha Govarthanan

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V. Sharma · B. E. Zipporah
Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamilnadu, India

S. K. Dash
Department of Mechanical Engineering, Indian Institute of Technology-Madras, Chennai, Tamil Nadu, India

P. K. Gupta
Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, Uttar Pradesh, India

Department of Biotechnology, Graphic Era Deemed to be University, Dehradun, Uttarakhand, India

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia

K. Govarthanan (✉)
MK BHAN Young Research Fellow, Centre for Cardiovascular Biology and Disease, Institute for Stem Cell Sciences and Regenerative Medicine (inStem), Bengaluru, Karnataka, India
e-mail: kavithag@instem.res.in

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Abstract

According to the latest figures released from American Heart Association, cardiovascular pathologies are the leading cause of morbidity and death, with MI as one with predominant cause of irreparable loss of the functional myocardium. Hitherto, the pharmacological intervention has not been in the forefront to ameliorate MI-related morbidity and mortality. Nevertheless, the contemporary therapeutic strategies can only delay the rate of disease progression but most of these fails to repair the damaged myocardium. Due to limited regenerative potential of the resident CSCs, there is a dire need to develop strategies that could regenerate the damage myocardium. However, the idea of using stem cells to differentiate into the cardiac lineage has so far been a relatively tough proposition for researchers around the world due to its complex nature. Scientist have explored stem cell-based tissue engineering/regenerative medicine strategies to regenerate the infarcted myocardium. Nonetheless, limited success was achieved in cardiac regenerative approaches due to the complexity of cardiomyogenic differentiation process, and usage of native decellularized ECMs and/or Matrigel support as a scaffold material. In this book chapter, we will discuss in detail various aspects of stem cell-based therapeutic strategies, which are currently being developed and employed for regenerating the damaged myocardium. The chapter contents highlight the basic technologies using scaffold-based injectable hydrogel or patch-based delivery system to the recent advanced technologies such as 3D Bioprinting approach to construct the cardiac tissue for cardiac regeneration applications.

Keywords

Cardiac · Hydrogel · Myocardial infarction · Scaffolds · Tissue engineering · 3D bioprinting

Abbreviations

2/3D	2/3-Dimensional
adeCM	Adipose Decellularized-Extracellular Matrix
ASCs	Adult Stem Cells
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Stromal Cell
CACs	Circulating Angiogenic Cells
CAD	Computer Aided Design
CD	Cluster of Differentiation
cdeCM	Cartilage Decellularized-Extracellular Matrix
CFs	Cardiac Fibroblasts
CMs	Cardiomyocytes
CNTs	Carbon Nanotubes
CPCs	Cardiac Progenitor Cells
CSCs	Cardiac stem cells
CVDs	Cardiovascular Diseases
CVS	Cardiovascular system

dECM	Decellularized Extracellular Matrix
ECM	Extracellular matrix
ECs	Endothelial Cells
EMT	Epithelial-Mesenchymal Transition
EPDCs	Epicardial-Derived Cells
FRESH	Freeform Reversible Embedding of Suspended Hydrogels
GAGs	Glycosaminoglycans
GATA-4	GATA Binding Protein 4
GelMA	Gelatin Methacryloyl
GFs	Growth Factors
hdECM	Human Decellularized Extracellular Matrix
IGF-1	Insulin like Growth Factor-1
iPSCs	Induced Pluripotent Stem Cells
kPa	Kilopascal
LVAD	Left Ventricular Assist Device
MI	Myocardial Infarction
MMP	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal Stem Cells
Nkx2.5	NK2 Homeobox 5
PCL	Poly (ε-caprolactone)
PEG	Polyethylene Glycol
PGA	Poly (glycolic acid)
PLA	Poly (lactic acid)
PLGA	Poly (lactide-co-glycolide)
PVA	Polyvinyl Alcohol
RGD	Arginine-Glycine-Aspartic acid
RTG	Reverse Thermal Gel
SDF1	Stromal Cell Derived Factor 1
SMCs	Smooth Muscle Cells
TERM	Tissue Engineering/Regenerative Medicine
TGF-1	Transforming Growth Factor 1
VEGF	Vascular Endothelial Growth Factor
WT1	Wilms Tumor 1
YIGSR	Tyrosine-Isoleucine-Glycine-Serine-Arginine
μCOP	Microscale Continuous Optical Printing

Introduction

CVDs are the leading cause of morbidity and mortality worldwide, responsible for nearly a quarter of all deaths and predicted to grow to 23.6 million by 2030 (Roth et al. 2015). According to the heart disease and Stroke update released from the American Heart Association, 2021, the prevalence of CVD (including heart failure, hypertension, stroke) in the population is 49.2% overall with age 20 years and above in the USA (Virani et al. 2021). Ischemia-related deaths have risen to the top of the list of causes of

death in India, the United States, and Europe (Prabhakaran et al. 2016; Benjamin et al. 2017; Timmis et al. 2020). The acute and chronic impact of CVD on individuals, communities, and healthcare institutions is enormous, particularly given the aging population and long-term unhealthy lifestyles. CVD is described as a group of conditions that harm the CVS and cause it to fail or have compromised functionality. The mortality is higher in CVDs and specifically post MI episode. MI progression ensues due to the pathological narrowing of the coronary vessels leading to the less than optimal blood flow to the heart. These pathological changes deprive the CMs of the much needed oxygen and nutrients thus resulting in massive CMs death. Resultantly, the ischemic tissue is replaced with a fibrotic scar as a part of the intrinsic repair mechanism thus entering the heart into a vicious cycle of remodeling (Fig. 1). Heart contractility and pumping capacity are hampered structurally and functionally as a consequence of adverse remodeling and loss of functioning myocytes. The situation is even more complicated due to the lack of a comprehensive, dynamic repair and regenerative ability of the myocardium. As a result, infarct expansion, ventricular dilation, and ventricular wall pressures are all factors that influence patients' morbidity and mortality (Reddy 2015).

While the available contemporary approaches of surgery and pharmacological intervention (i.e., vasodilators, diuretics) provide merely symptomatic relief, these approaches do not address the underlying root cause of the problem. End-stage heart failure patients receive a heart transplant, while high-risk patients receive LVAD supported by extensive long-term drug therapy. The post-MI scar is a priority to develop therapies that could help rebuild the ischemic myocardium (Rischpler 2016). Researchers have looked into different treatment options with the rise in CVD cases, and stem cell-based cardiac tissue engineering has been at the forefront. Stem cell-based therapy advanced to phase III clinical trials during the last two decades of research after extensive in vitro and in vivo experimentation. Regenerative medicine and tissue engineering are being combined to gain the better of the

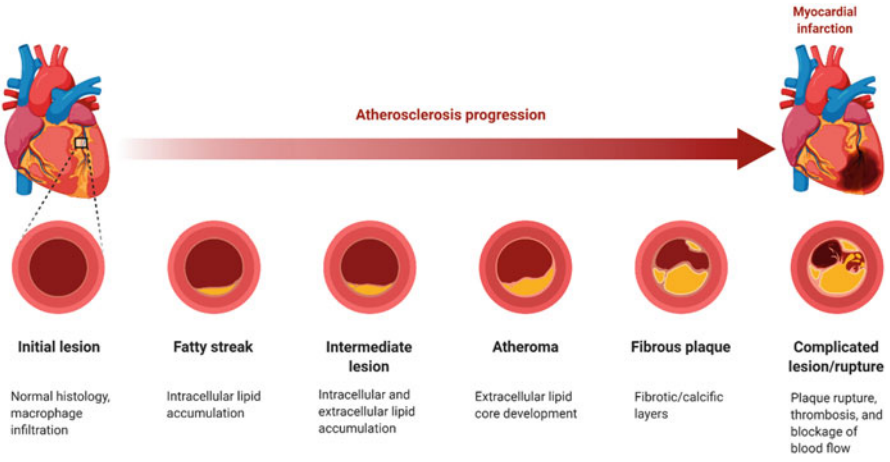

Fig. 1 Schematic progression of atherosclerosis

two fields to develop novel methods of therapeutic intervention. In this regard, researchers are experimenting with different combinations of cells, biomaterials, and bioactive molecules, which provide therapeutic benefits via multifactorial mechanisms. For example, the transplanted cells may transdifferentiate into morphofunctionally competent myocytes and integrate with the host tissue to assist in the repair process. Alternatively, the transplanted cells release paracrine factors causing endogenous stem/progenitor cells to respond in a reparative manner. Similarly, biomaterials could serve as adhesion sites for the transplanted as well as endogenous stem/progenitor cells and provide long-term release of bioactive molecules and signaling (Xu et al. 2016; Reis et al. 2016; Shafei et al. 2017).

Recently there has been immense interest in 3D-bioprinting to understand the mechanism of MI growth via mimicking the microenvironment and research the effect of drugs in the preclinical drug screening process (Cahill et al. 2017; Zhang et al. 2017b). This chapter discusses in-depth the latest developments in cardiac tissue engineering, emphasizing on the therapeutic approach for MI. Moreover, we have deliberated on the progress in 3D-bioprinting and its role in cardiac regeneration.

Engineering of Infarcted Myocardium

Although the idea of organ regeneration was first recorded in the seventeenth century in the form of a lizard tail, it was not until the nineteenth century that the regenerative repair of the human heart was seriously considered. Human heart has always been considered as a terminally differentiated organ with limited capacity to regenerate and repair itself. Although the presence of resident CSCs has been reported and there are also reports that CMs can re-enter into cell cycle in the event of myocardial injury, the intrinsic repair mechanism of the heart is inefficient especially in the event of massive CMs death. This is complicated further by the scarring that occurs in the infarcted region. Although the heart's scar formation and regenerative ability have yet to be fully characterized, it is thought that the mechanical properties of the myocardium are maintained despite a greatly reduced heart function (Cahill et al. 2017).

Cell-based regenerative therapy is being widely investigated to support the inefficient intrinsic repair mechanism of the heart to substitute the dead CMs with new myocytes, improve regional blood flow to the ischemic myocardium, and preserve or restore the global function of the heart post-MI. Different strategies, including delivery of stem cells either alone or combined with GF treatment or gene therapy, have been developed in this regard. These strategies are both preventative and curative, as they help in the recovery of lost CMs and support regenerating the infarcted myocardium as well as restoring regional blood flow (Vunjak-Novakovic et al. 2010). Various types of stem/progenitor cells have been characterized as potential candidates to treat the infarcted heart due to continued exploration of stem cell biology and the knowledge gained through it for its possible use in application-based research.

MSCs in Cardiac Regenerative Therapy

Dr. Julius Friedrich Cohnheim, a German pathologist, was the first to suggest the existence of non-hematopoietic stem cells in the bone marrow in 1867. Later, Freidenstein's cells were dubbed MSCs (Friedenstein 1976; Friedenstein et al. 1976), a term coined in 1991 by Caplan and colleagues, who suggested the existence of ASCs responsible for mesengenesis (Caplan 1991; Cui et al. 2018).

Based on Friedenstein's research, scientists discovered that bone marrow contains a subpopulation of MSCs that can differentiate into various cell types, including bone, adipocytes, and cartilage. Besides, MSCs have been successfully differentiated into a variety of other cell types in the last two decades, including CMs, hepatocytes, neurons, and ECs. Given their robustness and ease of genetic manipulation, they have also been used as carriers of exogenous genes of interest for delivery to the infarcted heart (Kim et al. 2012a; Ahmed et al. 2010; Haider et al. 2008; Jiang et al. 2008) besides their use as a starting material to develop iPSCs using Takahashi and Yamanaka's classical protocol of quartet of transcription factors (Buccini et al. 2012; Ibrahim et al. 2016).

MSCs constitute a heterogeneous groups of cells which can be identified based of their preferential adherence to the plastic surface, tri-lineage differentiation to adopt adipogenic, chondrogenic, and osteogenic fate besides the expression of a select group of surface markers, i.e., CD44, CD73, CD90, and CD105 and absence of CD34, CD38, CD45, CD11/14b (Haider 2018). This is the minimum criterion set by the International Society of Stem Cells to identify the purity of the MSCs. Various induction protocols have been developed and optimized to differentiate MSCs from multiple sources into CM-like cells. Some of the most commonly used approaches include treating MSCs with cardiac tissue extracts, co-culturing MSCs with CMs, or culturing with pro-cardiogenic factors (BMP, VEGF, TGF-1, angiotensin 2, or treatment with small molecules, i.e., 5 Azacytidine) (Ullah et al. 2021; Zeng et al. 2008; Perán et al. 2010; Guan et al. 2011; Markmee et al. 2017; Cui et al. 2018). Besides, MSCs have also been preconditioned at sub-cellular (Lu et al. 2010) and cellular levels to enhance their proliferation, functionality, paracrine activity, and reparability (Li et al. 2012, Haider et al. 2010; Kim et al. 2012a, b; Haider and Aziz 2017).

The field of cardiac tissue engineering has garnered much attention during the last decade. The first study on the cardiac tissue growth derived from embryonic chicken CM in vitro was published in 1995, which sparked a lot of interest. Thus, cardiac tissue engineering emerged from such pioneering studies to turn basic knowledge into clinical practice. The long-term objectives of these advancements have led to the development of small and large experimental animal models for preclinical evaluation of drugs, tissue constructs to regenerate myocardium in vivo, and better pathophysiological understanding of cardiac-related diseases (Feric and Radisic 2016).

Natural and Synthetic Polymer Used for Regenerative Therapy

Engineered functional biomaterials for cardiac therapies have emerged as a viable option to meet the global demand for novel CVD treatments. Biomaterials are 3D scaffolds made up of natural or synthetic materials engineered to replicate the local cellular microenvironment. The combination of TERM aims to restore damaged tissues' structural and functional properties. Biomaterials are used in cardiovascular applications to help or temporarily replace the damaged myocardial tissue and serve as a base for long-term repair and regeneration. For example, biomaterials contribute to the repair process by controlling the cellular and molecular pathways (i.e., inflammation and fibrosis), improving ECM and tissue integrity, and sustaining CM viability (Segers and Lee 2011; Reis et al. 2016; Gaharwar et al. 2020). Therefore, biomaterials should have optimal mechanical and biological characteristics that render them biocompatible, biodegradable, and biomimetic to meet the TERM requirements (Segers and Lee 2011). Cell seeding (either in vivo or ex vivo) onto implantable scaffolds or acellular scaffolds that recruit endogenous cells to promote reparative processes is currently used in biomaterial approaches to enhance therapeutic efficacy. Scaffolds are also being used for site-specific delivery of cells while concomitantly improving their rate of engraftment and survival (Terrovitis et al. 2010; Li et al. 2017; Su et al. 2018; Huang et al. 2020). They may be impregnated with cytokines or GFs that promote angiogenesis in cardiac tissues to improve regional blood flow (Lakshmanan et al. 2016). Akin to the healthy myocardial environment, the structural and mechanical properties of the scaffolds, i.e., anisotropic and topographic cues, can modulate CM and fibroblast morphology, distribution, and function (Silvestri et al. 2013; Sia et al. 2016). These scaffolds are usually made as cardiac pads, fibrous mats, macroporous sponges, foams, or hydrogels of various shapes and sizes. Furthermore, these characteristics are the primary determinants of the physical, mechanical, and biological properties of any material being used for a scaffold. Therefore, the biomaterials must be characterized, keeping in view the conditions (ECM, myocardial tissue) they would ultimately be used (Mackiewicz et al. 2016).

Natural Scaffolds

Natural-based scaffolds made of polysaccharides and/or proteins can be constructed to imitate the physical and biological characteristics of the intrinsic heart ECM. Collagen, fibrin, alginate, chitosan, and hyaluronic acid are commonly used to develop a typical natural scaffold. In general, these natural-source components are biocompatible and bioactive and significantly promote the biological characteristics of the cells, including cell adhesion, proliferation, differentiation, and paracrine behavior of the cells (Asadi et al. 2020). Furthermore, decellularized tissues that have undergone comprehensive pre- and post-processing retain their tissue-specific

characteristics that are essential for the scaffold material-host interaction with multi-layered biological networks containing non-cellular components. Together, this typical scaffold mimics the biological microarchitecture of the heart's dynamic native ECM (Li et al. 2020). On the contrary, natural scaffolds offer many challenges, including cost, poor stability and reproducibility, and relatively weak mechanical properties. Furthermore, due to the associated risks with the biomaterial preparation and origin (animal-based), immunogenicity remains a primary issue for their clinical translation (Pawan et al. 2019). We have discussed in-depth ECM and collagen-based scaffold for their significance in cardiac regeneration research.

Extra-Cellular Matrix (ECM)

The ECM plays an essential role in the differentiation of stem cells into a specific lineage. ECM-directed differentiation of stem cells has been intriguing because, in addition to providing a conducive matrix for cellular attachment, growth, and proliferation, its biochemical composition, and the presence of various GFs, significantly contribute to deliver differentiation-relevant biochemical cues for the seeded cells (Baharvand et al. 2005; Rienks et al. 2014; D'Amore et al. 2016; Pawan et al. 2019; Li et al. 2020). Emanating from different cells, including CFs, MSCs, and fibroblasts, the composition of ECM differs according to its cell source and significantly influences cardiomyogenic differentiation of cells. For example, a direct comparison of CF-derived ECM, cardiogel, with BMSC-derived ECM, mesogel, revealed that two ECMs diverged in their composition in the differential expression of nearly 17 ECM proteins (Santhakumar et al. 2014).

The distinct difference in their composition was related to their differential ability to promote cardiomyogenic differentiation and angiogenic potential of stem cells. The authors also reported that cardiogel also promoted proliferation, adhesion, and migration potential of the cells in culture. In addition, it protects CMs by reducing the oxidative stress on primary CMs (Sreejit and Verma 2011). The authors observed that MSCs adhered to the cardiogel at a much faster rate than MSCs cultured on the normal surface and were difficult to dislodge by proteolytic enzyme digestion besides showing enhanced survival under oxidative stress of treatment with H_2O_2. Others have also found that ECM obtained from MSCs protects C-kit positive CMs from oxidative stress (Ng et al. 2019). The experimental data showed that the addition of 5-Azacytidine, an epigenetic modulator commonly used to induce cardiomyogenic differentiation can improve MSCs' differentiation into CMs when the cells were cultured in CF-derived ECM (Santhakumar et al. 2014). This method could be helpful in the development of functional CMs and their eventual in vivo application for the treatment of experimentally induced MI. Such cell-derived ECM can be used to differentiate MSCs into CMs in vitro and in vivo (Santhakumar et al. 2014).

It is now well-documented that the ECM plays a critical role in the fate determination of MSCs during their cardiomyogenic differentiation. Decellularized heart ECM has been used as a natural scaffold for CMs culture and MSCs' differentiation into CMs during the construction of an experimental artificial whole organ. There has been a greater focus on the area of artificial whole organ development since the

publication of the first report to use the decellularized whole organ for artificial heart reconstruction (Ott et al. 2008). CMs obtained from MSCs have a better survival and migration rate in a decellularized heart scaffold (Akbay and Onur 2019). From the standpoint of building a large-scale artificial organ with a high cell density, this is a piece of important information. Repopulation of decellularized heart ECM and in situ differentiation have been used to make a whole reconstructed heart based on CMs. CMs obtained from rat MSCs, as well as rat primary CMs and ECs, were recently repopulated into decellularized rat heart. Although this construct demonstrated the repopulation of a decellularized rat heart, the repopulated heart's conduction was lacking (Tong et al. 2019). This research focused on one of the most intriguing aspects of obtaining morphofunctionally competent CMs from MSCs. However, such an approach necessitates more in-depth research to develop optimized differentiation protocols for overall repopulation, and impulse generation and propagation in the reconstructed heart. MSCs and decellularized heart matrix have been used in a similar manner to make a functional patch that can help alleviate or treat infarcted heart. This was accomplished by seeding MSCs derived from the adipose tissue into a decellularized porcine heart patch. This decellularized patch carrying the adipose tissue cells showed increased proliferation and infiltration, indicating that it could be used as a scaffold for cell transplantation and tissue construction. Transplantation of such a construct into a rat model of MI improved cell infiltration and increased vascularization at the infarct site (Shah et al. 2018). These approaches are intriguing because using heart-derived matrices would resemble the support scaffold more closely to the ECM conditions in the native heart tissue, optimally influencing the performance and outcome of the transplanted scaffold in improving the global heart function. Heart ECM-derived hydrogel has also been used to develop CM-like cells and their application in improving heart function in vivo. Heart ECM-derived hydrogel supported MSCs' proliferation and commitment to CM-like cells in this study. The therapeutic potential and application of ECM hydrogel as a model for cell delivery in heart repair following infarction were also validated in vivo (Bai et al. 2019).

Collagen Scaffolds

Collagen, in particular, has garnered a lot of attention as a viable natural scaffold for tissue engineering (Wu et al. 2019). Collagen is a fibrous structural protein that is present in fibrillar and non-fibrillar forms in most mammalian connective tissues and accounts for nearly 25% of total dry weight (Parenteau-Bareil et al. 2010). Collagens are distinguished by their triple-helical domain, which is formed by combining three polypeptide chains with Gly-X-Y repeat sequences to create a homo-trimeric or hetero-trimeric supramolecular structure. Glycine residues are found in every third position in the repeated triplet series, followed by hydroxylysine, hydroxyproline, or proline residues in the X and Y positions (Shoulders and Raines 2009; Parenteau-Bareil et al. 2010; Chattopadhyay and Raines 2014). Coiled turns in the polypeptide chains are possible with this sequence. The structural properties of collagen vary due to variations in the chain structure, but the helical shape in fibrillar collagens is conserved. Collagens are made and secreted as procollagen, primarily from

fibroblasts that have undergone post-translational modifications. The tropocollagen self-assembly creates fibrils that bind to collagen fibers on the outside (Chattopadhyay and Raines 2014). Furthermore, type I and type III fibrillar collagens are essential components of cardiac tissue's ECM. Co-polymerized type I and type III bundles make up the bulk of fibers surrounding CMs (Gazoti Debessa et al. 2001). These collagens arranged in three layers/bundles: epimysial, perimysial, and endomysial fibers provide structural and functional support to the surrounding cardiac tissues. The primary alignment and orientation of fibers in the collagen network provide a functionally active surface for cellular development, communication, and contractility (Shoulders and Raines 2009). Collagen motifs (e.g., RGD) also interact with various cell receptor proteins (e.g., integrins) and other ECM components. Adhesion, migration, proliferation, and differentiation are all supported by these explicit but indirect interactions. Collagens have outstanding biocompatibility and biodegradability properties of their own and are abundant and essential components of a healthy cardiac ECM structure and function (Kaiser et al. 2019). MMPs (e.g., collagenases) enzymatic activity also plays a role in collagen biodegradation. These properties can be modulated by cross-linking techniques (physical, chemical, and enzymatic) that shape polymeric networks in the development of collagen-based biomaterials (Parenteau-Bareil et al. 2010; Chattopadhyay and Raines 2014). These characteristics render collagen an ideal natural material for biomaterials; however, it has a few disadvantages. For example, the mechanical properties of collagens are highly conserved, which makes its optimization a problematic proposition, while the unmodified polymers behave less than optimally. A careful handling is also needed to avoid denaturation, degradation, or fibrillogenesis, (Shoulders and Raines 2009).

Notably, the two primary forms of ECM collagen, type I and type III are structurally different that significantly impact their function as well as mechanical properties in the heart. Collagen type I is the most abundant type that imparts strength and stiffness to the fibrous networks. On the other hand, collagen type III has a major regulatory role in myocyte function. It is interspersed in collagen type I meshwork to provide elasticity. The physical and mechanical properties of native and post-MI tissues are significantly influenced by the ECM ratio between the two collagen types. For example, in the diseased heart, type I is generally elevated relative to type III, whereas early-stage post-MI type III shows a dramatic rise, which results in impaired cell adhesion and decreased angiogenesis. On the same note, other pathological conditions, i.e., vascular Ehlers-Danlos syndrome, are affected by this fluctuating ratio-dependent phenomenon, which affects fibril covalent cross-linking variations between the two collagens. With the emergence of new fibers in the LV wall, collagen type I content rises and CMs number falls due to the advancing age of the patient. This is analogous to variations in ratios between uninjured and hypertrophic skin over time. Moreover, with less elastic type III collagen, the type I/III ratio changes, which may contribute to a proportionate decline in cardiac function. Type I fibrillogenesis, or fiber length/diameter, are affected by type III during co-assembly in the cardiac environment. Furthermore, in myocardial fibrosis, an increased collagen types I/III ratio has been linked to

dilated cardiomyopathy. Thus, the effect of collagen-based materials can be influenced by variations in myocardial collagen form I/III disposition at various stages of life or injury (Parenteau-Bareil et al. 2010; Chattopadhyay and Raines 2014).

The collagen-based scaffolds, especially those based on collagen type I, have long been a popular natural source for cardiac biomaterials. Collagen-based matrices enhanced vascularization and tissue repair in the ischemic and infarcted tissues. However, delivery time of the injectable collagen remains a critical determinant of the beneficial therapeutic effects of the treatment post-infarction (Blackburn et al. 2015). While injection of the collagen hydrogel showed long-term benefits in alleviating ventricular remodeling, delay in the treatment was less beneficial. On the mechanistic aspect, injectable hydrogel suppressed the ongoing inflammatory response in the infarcted myocardium by altering the cytokine profile, thus reducing tissue fibrosis and promoting angiogenesis. These data also showed that collagen materials are capable of more than just offering structural support. Regulated degradation of collagen materials has been shown in studies to improve GF retention and angiogenesis. Collagen matrices are excellent for cell delivery that supports cell recruitment for their participation in the naturally ongoing repair process, enhancing cell survival and tissue preservation. Collagen offers biophysical and biochemical cues that control cell behavior as an effective regenerative environment. Collagen also helps many stem cells and progenitors, i.e., CACs, MSCs, and skeletal myoblasts, to participate in the repair process (Kuraitis et al. 2012). There is strong evidence in the published data that functionality of the collagen scaffolds can be further enhanced by scaffold electrostimulation or decellularized hybrid materials that facilitate stem cell differentiation into CMs with pre-conditioned collagen scaffolds for cardiac phenotypes (Chen et al. 2019). Given their 3D environments, collagen hydrogels or soft matrices show the most positive cellular interactions (Hasan et al. 2015). For example, treatment of MSCs with collagen promotes their cardiomyogenic differentiation. Kang et al. showed that the rate of cardiomyogenic differentiation of MSCs cultured on nanosized collagen type I after treatment with 5-azacytidine was significantly higher than the routinely cultured cells treated with 5-azacytidine. In addition, the collagen-cultured MSCs showed significantly higher expression of Nkx2.5 and GATA-4 proteins besides cardiac-specific troponin-I, myosin heavy chain, and cardiac α-actin (Kang et al. 2013). Similar data was also published by other research groups (2013). Interestingly, the strategy of 3D culturing of the cells not only enhanced the rate of cardiomyogenic differentiation, the derived CMs also showed superiority in their functionality (Ding et al. 2020).

Although collagen is an excellent natural option for composite scaffolds, it can be enhanced by adding additional components such as polysaccharides or other ECM-derived proteins. Collagen also aids in cell attachment and differentiation in scaffolds made of synthetic materials like PCL, PLGA, and PLA. Collagen nanofibers infused with gold nanoparticles improved fiber conductivity and facilitated MSCs differentiation cultured in cardiac differentiation medium in the nanomaterials. Collagen biomaterials are also suitable for cardiac regeneration due to their mechanical and biological properties. Collagen scaffolds can be further refined

with novel design techniques, nanotechnology, and composites that are at the forefront of next-generation materials (McLaughlin et al. 2019).

Synthetic Scaffolds

Unlike biomaterials, synthetic material scaffolds inherently lack biocompatibility; however, they provide remarkable design and preparation flexibility and variability. Therefore, polyesters and polyurethanes are more commonly used for continuous, low-cost development of physical and mechanical properties that are easily customizable. In addition, some synthetics, such as PCL, PLGA, and PLA, are biodegradable, and the majority are easily modifiable (Gupta et al. 2021). However, foreign body reactions, toxicity, and undefined functional characteristics are all disadvantages of the design. Furthermore, synthetic scaffolds usually have surface hydrophobicity and, without alteration, lack internal or external biological components, which make cultivating interactions for cell growth and tissue organization difficult (Silvestri et al. 2013; Tong and Yang 2018).

Hybrid biomaterials incorporate natural and synthetic elements to create scaffolds with better structural and functional properties. Many different techniques, i.e., gas foaming, freeze-drying, electrospinning, are used to assemble a single scaffold in hybrid production methods. Natural elements are typically layered over synthetic elements, either in the form of multi-layered fibers or shell and core structures. Natural materials alter exterior bio-integration and surface interactions, while synthetics add strength to the mechanical properties or impart unique geometries to the scaffold. Cell orientation, permeation, contractility, and movements are all influenced by compartmentalized or whole material geometry (e.g., form, scale, porosity, fiber dimensions). Electrospun fibrous scaffolds or self-assembling peptides are typical examples of this. However, thorough characterization is needed for composite or hybrid biomaterials to ensure that the individual properties imparted by each component are preserved during pre- and post-processing methods (Bagno et al. 2018).

To enhance their structural and functional properties, biomaterials may be modified morphologically, chemically, or biologically during the manufacturing process (e.g., cross-linking, bioactive molecules) or afterward (e.g., surface functionalization). For example, specific functional groups or cell-binding motifs (RGD, YIGSR) used on surface coatings improve cell adhesion (Silvestri et al. 2013). Laser-enhanced micro-channels that boost cell attachment and retention can be used to modify scaffold surfaces with utmost precision. Impregnation of the scaffolds with GFs promotes regenerative processes in the cardiac tissues and includes VEGF and IGF-1. Covalent fixation to surfaces, encapsulation, or cross-linkage may all be used to attach GFs to scaffolds. Composite scaffolds, especially hydrogels, easily expose GFs to tissues due to their unique localization and time-release degradation. These changes support cell survival and mediate cell development, proliferation, and differentiation.

Hydrogel Based Therapy

Because of their crosslinked 3D network, which can contain up to 98% water and mimic the native ECM, hydrogels are a natural option for many regenerative medicine applications. As a result, hydrogels are being created for cell and drug delivery, as well as for use as bioactive agents to aid healing and tissue regeneration following injury or disease. Hydrogel designs for the treatment of trauma and diseases of the CVS, nervous system, cartilage, bone, skin, and cornea, to name a few, have been published (Toh and Loh 2014; Floren et al. 2016; Brunette et al. 2017; Dimatteo et al. 2018).

Each application of hydrogel necessitates a particular set of physical and biological properties during its development for biomedical use (Fig. 2). Viscoelasticity, shear-thinning, self-healing, and thermal properties are some of the specific examples of physical properties that need optimization for ideal usefulness and maximum benefits. In addition, the hydrogels must be non-toxic from the biological perspective. To achieve the required biological response, biodegradability properties, their ability to provide adequate space for cells, and the necessary cell attachment sites or other signaling factors (broadly defined as biocompatibility) require careful consideration (Floren et al. 2016). It is pertinent to mention that the physical properties offered by the scaffold in terms of cellular microenvironment have a significant bearing on the cell biology, their paracrine behavior, and differentiation characteristics.

For example, the effects of substrate stiffness on various cell types under diverse set of conditions are enormous and have been extensively studied in vitro experimental settings. CMs, for example, continue to beat on the substrates that mimic the

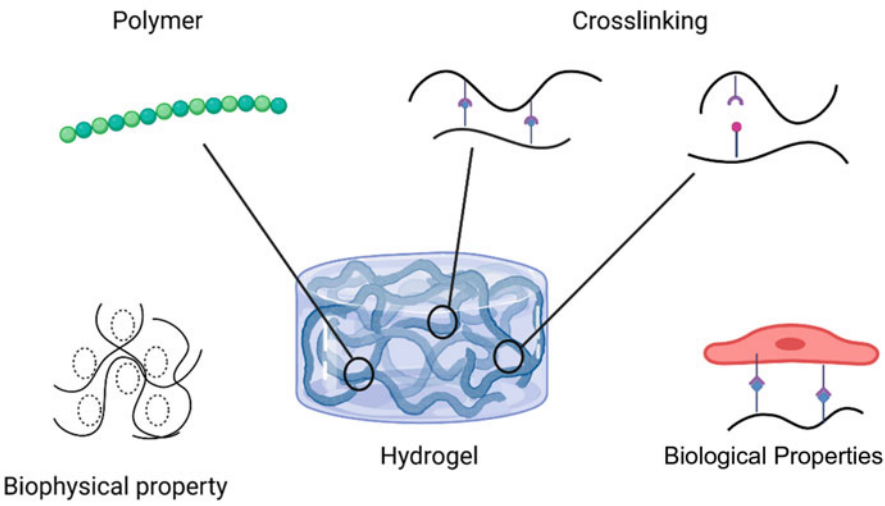

Fig. 2 Some of the key factors to be considered during the designing of a hydrogel system

measured stiffness of healthy myocardium. In contrast, the substrates with a higher stiffness corresponding to a MI scar failed to support their contractility.

TERM applications, which often use stem and progenitor cells in conjunction with hydrogels, require the sustenance of stem cell activity after their culturing on different substrates. For example, the elastic modulus of a polyacrylamide hydrogel directs MSCs to undergo neurogenic, myogenic, and osteogenic differentiation from low (1 kPa) to medium (10–20 kPa) to high (100 kPa) (Daviran et al. 2018). Similarly, $CD34^+$ cells from mouse bone marrow successfully adopted endothelial fate on a substrate matching myocardial tissue stiffness 1–2 weeks after the MI (Zhang et al. 2017a). However, in vivo environments, where cells are embedded in a 3D matrix, the 2D cell culture environment may not be entirely representative. When the hydrogels must be crosslinked in the presence of cells using bio-orthogonal reactions, preventing cross-reaction with cell surface components, in that case, the development of 3D in vitro systems becomes a challenge. For example, alginate, hyaluronic acid, and gelatin hydrogels have been used to demonstrate that neural stem and progenitor cells undergo neuronal differentiation at a higher rate on the very soft hydrogels that match brain tissue stiffness.

In contrast, slightly stiffer hydrogels result in increased astrocyte differentiation (Zhang et al. 2017a). In summary, when selecting a hydrogel for tissue regeneration, the mechanical properties of the tissue should be taken into consideration and mimicked for the desired and best outcomes.

Hydrogels have viscous fluid properties (loss modulus, G) in addition to rigid elastic nature (storage modulus, G). This means that a portion of the energy is dissipated as heat during hydrogel deformation, resulting in a nonlinear time-dependent response to the applied stress (Özkaya et al. 2017). Thus, measuring the complex viscoelastic properties of hydrogels is arguably the best way to evaluate their response to deformation in general, and particularly given that the viscous characteristics of the environment have also been shown to affect cells. For example, MSCs' transdifferentiation into SMCs improved when cultured on higher viscosity substrates ($G = 130$ Pa vs. 1 Pa) while retaining the same storage modulus (5 kPa). Furthermore, both in vitro and in vivo, stiffness-matched ($G = 20$ kPa) hydrogels with quick stress-relaxation (more pronounced viscous character) resulted in MSCs' improved rate of osteogenic differentiation (Cameron et al. 2014; Özkaya et al. 2017; Fan et al. 2018).

About the intended use, the choice of polymer, crosslinking process, physical and biological properties of the hydrogel, and their characterization should all be considered in unison for a collective perspective. Fortunately, the idea of a hydrogel allows room for lot of creativity, especially when it comes to creating a material that meets the requirements of a specific application. Synthetic or natural polymers, or a mixture of both, may be used to make hydrogels. Different chemical reactions or physical interactions may be used to build the crosslinked 3D network. However, the development of a universal hydrogel design is implausible to be achieved because each material and its crosslinking process has characteristics that could be advantageous or disadvantageous depending on the intended application; instead, it must be adapted to each particular biomedical situation (Toh and Loh 2014).

Injectable hydrogels, both natural and synthetic, are hydrated solutions that solidify in response to physiological pH, temperature, and chemical cross-linking. They are water-absorbent, malleable, and durable soft materials that shape complex, hydrophilic 3D environments. Environmental biomimetic applications benefit from the high-water retention and polymeric network, which is also improved by covalent cross-linking. Surface adhesion and permeability are enhanced by hydrogels' structural expansion, including water retention due to the porous networks. This allows the seeded cells to communicate with each other and with the substrate more easily, offering both physical and functional support. Furthermore, the spatial organization of solidified hydrogels acts as a scaffold, enhancing transplanted cell retention, biological characteristics, and functionality. Furthermore, in situ gelation is a beneficial property of hydrogels because, as an injectable, the delivery mechanism reduces invasive procedures and allows for easier delivery as compared to rigid materials such as tissue patches or delicate decellularized sheets that not only require special handling in vitro but also obligate more invasive procedures for site-directed delivery/placement.

In the experimental animal models, injectable materials have demonstrated the potential to increase cardiac function and reduce LV-dilation even without exogenous cells. However, the mechanisms that promote functional regeneration and tissue neovascularization through material-cell-host interactions remain less well-understood and have yet to be thoroughly elucidated. In reality, the structural support offered by an inert material has been shown to have little or no inherent effect. As a result, the focus of research is on searching for clinically more viable materials and understanding their bioactive interactions in the cardiac system (Hao et al. 2007; Hasan et al. 2015; Ruvinov and Cohen 2016; Ketabat et al. 2018; Matsumura et al. 2019).

Patch-Based Cell Therapy

Though hydrogel-based therapy has given encouraging data regarding safety and efficacy, various research groups have focused on site-specific delivery of cells for infarct treatment using a patch-based cell delivery approach. 3D printing of pre-vascularized and multi-material structures uses decellularized ECM carrying stem cells as bio-inks. The printed structure, which consists of dual stem cell spatial patterning, improves cardiac functions by attenuating cardiac hypertrophy and fibrosis, increasing migration of the delivered cells from the patch to the infarct region. Patch-based delivered cells significantly contributed to neovascularization, resulting in improved global cardiac function. Jang et al. have reported an interesting study in which stem cell-carrying hdECM bioinks were used in 3D printing or multi-material structures (Jang et al. 2017). The spatial patterning of the stem cells enhanced their differentiation potential. The stem cell carrying patches were allowed the migration of WT1 positive progenitor cells using the EMT mechanism, hdECM enhances epicardial-mediated cardiac tissue regeneration in an experimental animal model (Jang et al. 2017).

The fabricated cardiac patches need invasive open-heart surgery for implantation, either by suturing (Shadrin et al. 2017) or applying bioglue (Singh et al. 2016). In

hypoxic and serum-deprived conditions, PCL/gelatin patch carrying MSCs stimulated endogenous cardiac repair. Mechanistically, increased MSCs survival and the paracrine release of VEGF, and SDF-1 contributed toward cardiac repair. Tracing engrafted MSCs fate for survival and distribution revealed that the cells were spread across the epicardium and penetrated into the myocardium. EPDCs migrated into deep tissue and differentiated into ECs and SMCs, with a low rate of cardiomyogenic differentiation (Wang et al. 2017). The delivery of non-suturable MSCs-carrying adhesive dressings has also exhibited regenerative potential. In a study by Kobayashi et al. (2019), adhesive dressing incorporated with MSCs was fabricated from a fibrin sealant film at the treatment site. The dressing firmly adhered to the surface of the heart at the site of application. The outer collagen film protected the MSCs-fibrin complex from erosion, supporting improved cell retention and migrating the delivered cells to the epicardium. The authors observed a 17% increase in the global LV function. The fibrin patch with a combination of thrombin is attributed to the adhesive property of the patch (Kobayashi et al. 2019).

As described earlier, the cardiac patch needs an application to the myocardium under direct vision, which is only possible by open-chest surgery, an invasive intervention. Therefore, the patch-based delivery approach has been modified to make it less invasive and clinically more relevant for routine applications. Biospray approach was reported by Tang et al., who used platelet-fibrin gel "paint" that polymerizes in situ with a minimally invasive procedure. They found that spray therapy after the MI episodes increased cardiac recovery and alleviated global cardiac dysfunction (Tang et al. 2017). On the same note, Montgomery et al. produced elastic and micro-fabricated scaffold that could be injected instead of delivered as a patch. After delivery via a 1 mm orifice, the scaffold regained its original form while retaining the viability and functionality of the delivered cells. In an experimental rat model of MI, the significant improvement in the global cardiac function was observed after delivering a micro-fabricated scaffold and cardiac patch of a biodegradable polymer (Montgomery et al. 2017). The approach required a minimally invasive cell delivery procedure. Pea et al. have also reported an injectable RTG-based scaffold functionalized with CNT, which transitions from a solution at room temperature to a 3D gel-based matrix shortly after reaching the body temperature, supporting long-term CMs survival, promoting CMs alignment and proliferation, and improving CMs function. The addition of CNTs to the RTG provided the native CMs with topographical and electrophysiological cues, facilitating long-term survival through better cell alignment and fibroblast proliferation suppression (Peña et al. 2017).

3D-Bioprinting

There are diverse strategies to repair the damaged area to improve the organ's functionality during experimental tissue regeneration. For example, the agent for damaged tissue repair may require implantation on the damaged tissue site, or it may require a mixture of cells and materials that will help regenerate the damaged part.

The materials and the cells will depend on the type of the target organ. For organs like the heart, which remain in constant motion, it is challenging to choose them owing to their dynamic functional status. Moreover, the types of cells seeded will determine their arrangement in the matrix, the mechanical property of the matrix, etc., all are even more important to achieve the desired outcome. In such a situation, 3D-Bioprinting is beneficial as it is easier to mimic the microenvironment of such complex tissues (Zhang et al. 2017b).

The Complexity of the Cardiac Tissue

The adult mammalian heart is intricate as it is hierarchically organized with multiple layers of cells in its wall. The heart's pump function is attributed to the concomitant and homogeneous contractility of the constituent contractile CMs that function as a syncytium. Each CM is around 120 μm long and joins with adjacent CMs to form a functional syncytium. Each CM is surrounded by connective tissue called endomysium, and a bundle of CMs is covered with perimysium connective tissue. Both of them have the function of providing support and preventing slip between cells. These bundles of CMs are arranged in layers with different alignments along the thickness of the heart. Deep inside the heart, they are arranged longitudinally; in the middle portion, the arrangement is circumferential, whereas the superficial strands are oblique (Ho 2009). This arrangement of myocardial strands is necessary for the generation of 3D heart contraction and effective systolic ejection of the blood. Recreating this anisotropic, multiscale architecture is crucial if CMs are used as the basic contractile unit for an engineered heart that can pump efficiently. CMs constitute more than 70% of the heart volume and nearly 30–40% of the total cell population in the heart (Khademhosseini and Camci-Unal 2020). The remaining cell populations include non-CMs, like fibroblasts, ECs etc. They perform important functions like mechanical stability, ECM secretion, nutrient transport, etc.

Balancing of these cell populations is crucial in 3D printing of the heart tissue for optimal function. Just like the cell types, several ECM proteins play a vital role in heart function, tissue organization and stability, i.e., collagen I, elastin, GAGs etc. Besides CMs and their support from ECM, heart has intricate group of structures like the heart valves, the coronary blood vessel system, and the cells responsible for conducting the electrical impulse throughout the cardiac tissue etc. as shown in Fig. 3. All these factors must be taken into consideration during engineering of the heart tissue.

3D-Printing Salient Points

3D printing was introduced in the late 1990s and has the capability to form scaffolds with a greater degree of architectural control. Encapsulation of cells with the biomaterials has made bioprinting a possible avenue. The only class of materials capable of maintaining cell viability during encapsulation is hydrogels. While

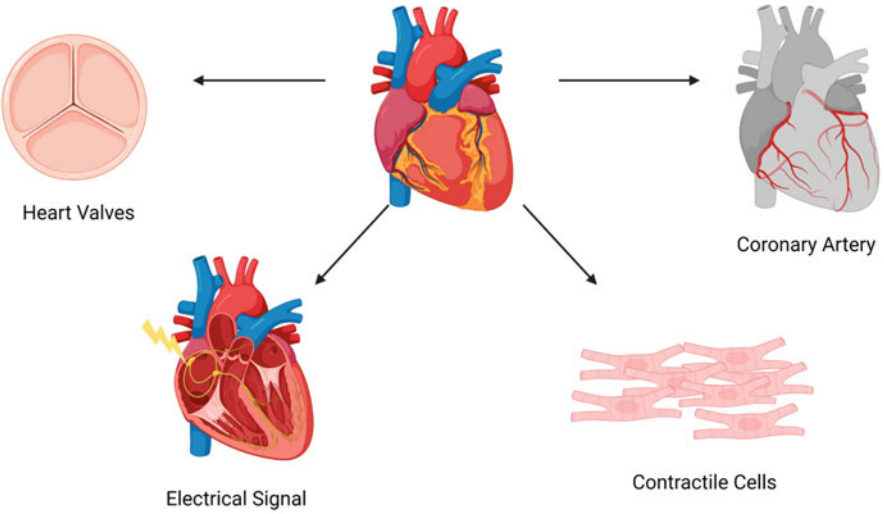

Fig. 3 Important structures and physiology of heart tissue, which requires to be replicated in 3D printed heart

making a new ink for 3D bioprinting, a compromise has to be made between its printability and cell viability. For example, inks with high polymer concentrations are the most amenable to 3D printing, but cell viability is best at low polymer concentration. Liquid phase bioinks apply less stress on cells while printing through extrusion, but they also have many problems, such as settling of cells during printing. Hence, it is not to print them in multiple layers, and they will need supporting structures or additional crosslinking mechanisms to achieve this. Gel-phase bioinks overcome such problems, but stresses on cells become an issue in them. Crosslinking of bioinks is a significant step in bioprinting.

It can be done by physical method (e.g., ionic bonding, hydrogen bonding, and hydrophobic forces) or chemical methods (e.g., photopolymerization) based on the properties of the native tissue. For example, ionic cross-linking is vulnerable to dissolution in culture or in vivo, where local divalent cation concentrations may draw cross-linking calcium out of the gel. So, the crosslinking method must be optimized before going for bioprinting. It should be noted that both physical and chemical crosslinking mechanisms can be combined to obtain a bioink. Tissue specificity may be achieved using tissue-specific dECM or by functionalizing a nonspecific ink with tissue-specific GFs. It is also required that the bioinks and their degradation products should not be cytotoxic. Naturally obtained bioinks have advantages as they can easily mimic the in vivo mechanical properties, cell viability and signaling is better than synthetic bioinks. Still there are issues of immune response, batch to batch variation associated with them. Some of the protein-based natural bioinks are collagen, gelatin, fibrin, silk, etc. Some of the polysaccharide-based natural bioinks are chitosan, hyaluronic acid, alginate, etc. Other natural

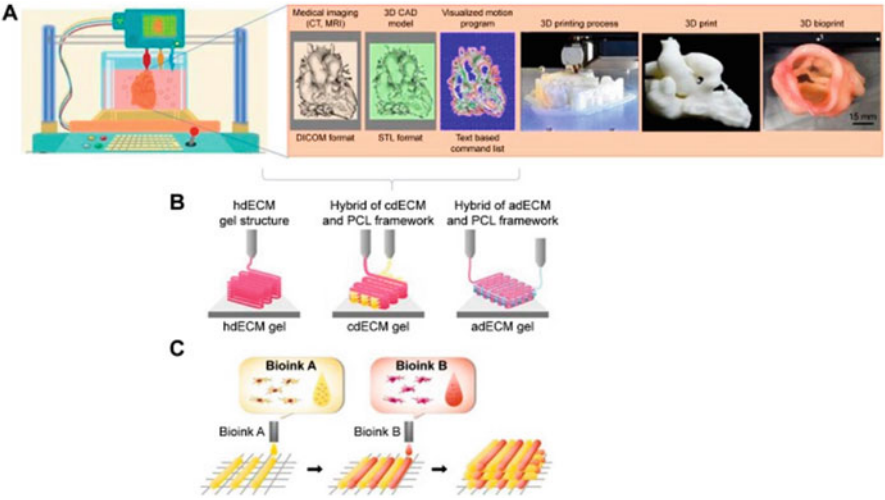

Fig. 4 Process of 3D-Bioprinting, (**a**) steps of 3D bioprinting, (**b**) pre-scaffold fabrication bioprinting, (**c**) simultaneous hybrid 3D bioprinting

bioinks include matrigel, decellularized ECM hydrogels, etc. Synthetic bioinks can be made from PEG or its mixture with other chemicals like Laponite. There are different methods for bioprinting too, such as inkjet printing, extrusion-based printing, or laser-based printing.

These methods vary in their capability to print a 3D structure laden with cells. For example, laser-based printing method has very high resolution and can print up to single cell resolution. Figure 4 shows different processes of 3D bioprinting of heart tissue.

3D Printing Techniques of Heart Tissue

The whole neonatal heart develops from the CPCs while the adult heart possesses limited capability to grow further and regenerate due to CMs senescence. This limited capacity also delimits its capacity to repair itself after pathologies like MI that lead to massive loss of functioning CMs and therefore necessitates outside intervention support. Various research groups have tried generating heart tissues and neovascularization form ESCs or iPSCs, or direct reprogramming from CFs (Rufaihah et al. 2007, 2010; Ahmed et al. 2011; Buccini et al. 2012). Though the transplanted cells successfully form beating CMs, the rate of differentiation is low and their alignment is improper with little evidence about their integration with the host myocytes, which is important requirement their harmonious functioning with the host myocardium. Given that electrical stimulation can direct cell orientation and migration whereas mechanical signals can induce cell alignment, forces like cyclic stretching may support the cell alignment concurrent with the direction of minimal

deformation rate. In terms of cell alignment, 3D printing supersedes other conventional methods for tissue engineering as it alleviates the need of external stimulus to induce cell alignment as cells can be printed in that fashion. Support-assisted bioprinting can also help overcome such issues with ease. Materials like Pluronic F127 can be used for the support structure which can be removed in later stage.

Printing of cell-embedded bioink can be performed in multiple layers to mimic the in vivo arrangement of cells in the heart. Such techniques also facilitate higher print resolution (Lee and Yeong 2020). Sacrificial material like PVA, which is easily soluble in water, can form a 3D structure. A valentine-shaped heart with micro size pores for nutrient transport has been performed using PVA. After sacrificing the PVA, cells in the hollow heart proliferated and maintained their biological properties (Zou et al. 2020). Purkinje fibers conduct cardiac action potential more quickly than other types of cells in the heart. It is necessary to include structures, which mediates CM polarization and depolarization to obtain a fully functional heart. Similarly, it is possible to print such a complicated system with the help of computational modeling and bioprinting with support structures, The current study assessed the functional performance of the Purkinje networks and continuous syncytium formation via response to electrical and biochemical stimulation (Tracy et al. 2020).

For transplantation of structures like a coronary artery, vascular grafts are used made out of biocompatible materials. When fabricated through conventional methods, they mostly fail because of mismatched vessel properties, thrombosis, atherosclerosis, or infection (Lee et al. 2019b). The physiology of blood vessels varies from patient to patient. Through computational modeling, patient-specific vascular grafts can be designed. 3D printing can help realize such vascular grafts with complex structures. For example, the Y-shaped graft, known to reduce power loss and optimize hydrodynamic efficiency, is relatively easier to print than any other method, including molding or electrospinning.

Another novel technique in 3D printing is μCOP that involves 2D pattern transformed into 3D volume. A higher resolution in the z direction with high-speed printing can be achieved with such techniques. Direct patterning of CMs in an asymmetric manner can be performed easily. Such systems can be used for both tissue engineering and drug discovery studies (Liu et al. 2020). New techniques have also been developed by different groups such as FRESH printing. The technique is based on the use of soft biomaterials for effective printing. From patient-derived MRI images, 3D printing of whole human heart is possible with this method using alginate hydrogel. The printed structure is strong enough to withstand the pulling force applied during suturing. Cell-laden FRESH printing is also possible (Mirdamadi et al. 2020). Drop-on-demand bioprinting technique can be used to print multiple layered structures like blood vessels. Blood vessels are composed of different layers, and in each layer, there are different types of cells and ECM composition. These 3D vessel models have a wall thickness of up to 425 μm and a diameter of about 1 mm. The models were successfully tested under physiological flow conditions for 3 weeks (Schöneberg et al. 2018). This technique can also be used for printing the complex structure of heart wall.

Most cells do not survive in human body when present in more than a few hundred micrometers from the nearest capillary due to nutrient deprivation. Hence, this should be kept in mind that 3D printing facilitates sufficient nutrient and oxygen transport to support cell survival. Also, the issue of nutrient transport can be solved by printing cell-laden bioinks in layers with spacing. To supply oxygen to the transplanted tissue, bioinks can be prepared with oxygen-generating materials, such as sodium percarbonate, calcium peroxide, magnesium peroxide, and hydrogen peroxide. The addition of calcium peroxide as an O_2 source in GelMA bioink improved cell survival under hypoxic conditions. Such materials also tend to change the viscosity of bioinks, which is needed to be considered while 3D printing (Erdem et al. 2020).

Bioinks for Heart Tissue

In addition to the cells, ECM is constantly changing during cardiac development to support cellular and organ needs. They are also of equal importance as cardiac cells as they are responsible for signaling and support. Their compositions vary with the age of the person. For example, in neonatal hearts, fibronectin is the most prevalent ECM while its percentage is meager in the adult heart wherein ECM is mainly composed of collagen I. ECM composition also varies with space and the percentage of proteins, i.e., elastin, fibrillin, or hyaluronic acid, change in atrium and ventricles. The bioink must contain the required ECM composition to support the growth and function of 3D printed tissue.

It is improbable that all these functions can be accomplished with a single bioink. Instead, it is needed to print a cardiac construct with multiple bioink formulations. Collagen is the most abundant matrix protein found in tissues. Conventionally, it is used for printing 3D scaffolds. With novel FRESH technology, it can be successfully used for fabricating heart tissue (Lee et al. 2019a). A combination of collagen with alginate or agarose has also been used to print 3D tissues with composite bioink (Gungor-Ozkerim et al. 2018). Nonnative structural materials like alginate or PEG alone have been used for bioprinting, but these materials do not support any biological function. They can only be used as structural support. Gelatin is another ECM component used for 3D printing the heart. The advantage of using gelatin is its intrinsic Arg-Gly-Asp (RGD) motifs, good biocompatibility, and low cost. Synchronized beating and proper alignment can also be achieved in gelatin bioink (Tijore et al. 2018). Other natural materials like chitosan and fibrin or synthetic materials like PGA, PCL, PLA, and PLGA has also been used for heart tissue engineering (Qasim et al. 2019). They have good biocompatibility but vary in cell migration capability, mechanical strength, vascularization, and cell adhesion. A combination of various available bioink materials can give rise to a novel bioink that will perform tissue repair in a better way. For example, in a recent work, different alginate-gelatin blends were constituted and tested for properties, like cell viability of MSCs, printability mechanical strength, etc. (Di Giuseppe et al. 2018). The authors showed that a blend of 7% alginate and 8% gelatin yields a bioink with high viability for

MSCs, printability, and mechanical strength. This bioink can be superior for the 3D printing of heart as MSCs are a cell source for cardiac tissue constructs.

Recently, matrigel has emerged as a novel bioink for cardiac tissue printing. It is a protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, an ECM mixture that also contains many factors like type IV collagen and sulfated proteoglycans. It can be formed into a hydrogel for the 3D printing of cardiac cells. It has been found that a hydrogel made of a mixture of collagen I and matrigel performs better for the culture of valve interstitial cells. It has better mechanical properties, comparable viability, cell phenotype, and proliferation with hydrogel made only out of collagen I (Lam et al. 2017). Matrigel enhances CM alignment and increases contractile stress too (Khademhosseini and Camci-Unal 2020). A hydrogel is formed from heart ECM after decellularization to obtain a bioink with properties close to an adult heart. Since they are derived from native tissue, they form an ideal bioink with tissue-specific biomolecular compositions (Abaci and Guvendiren 2020). The disadvantage of dECM is that it has weak mechanical properties. But this can be overcome by adding structural materials like PCL in the 3D printed tissue or developing a photo cross-linkable dECM. In recent work, such a bioink, has been formulated, which enhances CMs viability compared to collagen I control (Yu et al. 2019). It was also possible to tailor the modulus of the bioink and microarchitecture in the construct.

Conclusion and Future Directions

Though there are different bioinks and cell types that can be used to form a 3D printed heart, it has to be kept in mind that the heart is not a homogeneous mixture of ECM and cells. Different structures form a complete heart, akin to the dense CMs population developing a myofibril, and the connective tissue covering above it (endomysium and perimysium). Complex structures can be printed easily by the new-age 3D printing, but we have to go a long way to print a whole heart with in vivo-like structure, structural organization, and function.

Cardiac regeneration after MI is critical for the proper functioning of the heart. Cell-based therapy has been at the forefront for regenerating the damaged myocardium. However, there are limitations in the therapeutic efficacy of cell-based therapy due to the hostile nature of infarct. To overcome the aforementioned limitations, various delivery strategies have been explored for better cell retention. Thus, scaffold in the form of patch or hydrogel plays a crucial role in cardiac regeneration after MI. In addition, 3D printing has been widely used for generating cardiac constructs and organoid models for cardiac regeneration and a better understanding of drug toxicity.

Cross-References

► Bioengineering Technique Progress of Direct Cardiac Reprogramming
► Current State of Stem Cell Therapy for Heart Diseases

► Molecular Signature of Stem Cells Undergoing Cardiomyogenic Differentiation
► Stem Cell Applications in Cardiac Tissue Regeneration
► Unraveling the Mystery of Regenerative Medicine in the Treatment of Heart Failure

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Vascular Functional Recovery and Reparation by Human Endothelial Progenitor Cells

26

Alexander E. Berezin and Alexander A. Berezin

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Abstract

Endogenous vascular reparation is based on mobilization, differentiation, and proliferation of bone marrow–derived and resident proangiogenic endothelial precursors, which directly and indirectly participate in adaptive and maladaptive vascular remodeling processes. The endothelial progenitor cells (EPCs) being under epigenetic control, metabolic stimuli, and paracrine/autocrine regulation frequently demonstrate a weak ability to survival and lowered potency to migration and proliferation among patients with known cardiovascular disease. This phenomenon is known as EPC dysfunction, which is in the core of an altered endogenous repair system. Lowered levels and impaired functional abilities in both resident and circulating endothelial progenitors are considered crucial contributors of vascular remodeling and endothelial dysfunction, which are central players in the development and progression of atherosclerosis, arterial and pulmonary hypertension, myocardial infarction, and heart failure. Although there is

A. E. Berezin (✉)
Internal Medicine Department, State Medical University, Zaporozhye, Ukraine

A. A. Berezin
Internal Medicine Department, Academy of Post-graduating Education, State Medical University, Zaporozhye, Ukraine

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wide-range evidence of the fact that injections of exogenous bone marrow–derived proangiogenic endothelial precursors do not engraft into impaired vessels, but that such circulating EPCs may regulate vascular repair through several paracrine mechanisms and thereby attenuate clinical condition of the patients and possibly improve prognosis. The circulating secretome and extracellular vesicles released by the EPCs contain various proangiogenic factors (active peptides, lipids, transforming growth factor-beta, bone morphogenetic protein-2, matrix metalloproteinases, chromatin, and noncoding RNAs) that are crucial for spontaneously formed organized cell clusters to support proliferative response in surrounding tissues. Additionally, EPCs secretome maintains angiogenesis, neovascularization, immune response, and inflammation in injured vasculature. Previously supply of exogenous proliferative EPCs had been derived from various pluripotent stem cells such as embryonic stem cells or induced pluripotent stem cells, but so far they can be constructed from individual's reprogrammed fibroblasts and this procedure did not require inducing pluripotency and had lowered teratogenic risk. Moreover, there are great opportunities to stimulate resident EPCs to (trans)-differentiation and proliferation by exogenous extracellular vesicles embarked on appropriate active molecules and noncoding RNAs. Overall, the concept of support of vascular reparation through engrafting EPCs appears to be promising. The chapter depicted previous evidence of mechanisms of regulation of vascular repair by residence regenerative vascular cells and results of preclinical and clinical studies for vascular functional recovery and reparation by EPCs and extracellular vesicles.

Keywords

Endothelial progenitor cells · EPCs · Secretome · Vascular regeneration · Biomarkers · Myocardial infarction · Cardiomyopathy · Heart failure

Abbreviations

AE	Adverse effect
Akt1	RAC-alpha serine/Threonine-protein kinase
AMI	Acute myocardial infarction
CABG	Coronary artery bypass grafting
CXCR4	C-X-C chemokine receptor type 4
EF	Ejection fraction
eNOS	Endothelial NO synthase
EPCs	Endothelial progenitor cells
HF	Heart failure
HFrEF	Heart failure with reduced ejection fraction
HIF	Hypoxia-inducing factor
Hox	Transcriptional modulator of cell-cell and cell-extracellular matrix adhesion
HSCs	Hematopoietic stem cells
ISCT	International Society for Cellular Therapy

LV	Left ventricle
MACE	Major cardiovascular event
MI	Mocardial infarction
MMP	Matrix metalloproteinase
MUC	Melanoma cellular adhesion molecule
NO	Nitric oxide
PECAM	Platelet/Endothelial cell adhesion molecule
RNA	Ribonucleic acid
SCFR	Mast/Stem cell growth factor receptor
SDF	Stromal cell–derived factor-1
TGF-beta1	Transforming growth factor-beta 1
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor

Introduction

Endogenous vascular reparation is based on mobilization, differentiation, and proliferation of bone marrow–derived and resident proangiogenic endothelial precursors, which directly and indirectly participate in adaptive and maladaptive vascular remodeling processes. The endothelial progenitor cells (EPCs) being under epigenetic control, metabolic stimuli, and paracrine/autocrine regulation frequently demonstrate a weak ability to survive and lowered potency of migration and proliferation in patients with cardiovascular disease. This phenomenon is attributed to EPCs' dysfunction, which is in the core of an altered endogenous repair system. Low levels in number and impaired functional capacity, in both resident and circulating endothelial progenitors, are considered as crucial contributors to vascular remodeling and endothelial dysfunction besides having a central role in the development and progression of atherosclerosis, arterial and pulmonary hypertension, myocardial infarction, and heart failure (Berezin et al. 2015).

Despite there is wide range of evidence that the injection of exogenous bone marrow–derived proangiogenic endothelial precursors do not engraft into impaired vessels, the transplanted as well as the circulating EPCs participate in the vascular repair through several mechanisms, i.e., paracrine mechanisms, and thereby alleviate the clinical conditions of the patients and possibly improve their prognosis. The EPCs-derived secretome and extracellular vesicles contain various proangiogenic factors (active peptides, lipids, transforming growth factor-beta, bone morphogenetic protein-2, matrix metalloproteinases, chromatin, and noncoding RNAs) that are crucial for spontaneously formed organized cell clusters to support proliferative response in surrounding tissues. Additionally, EPCs secretome maintains angiogenesis, neovascularization, immune response, and inflammation in injured vasculature (Haider et al. 2017). Previously, supply of exogenous proliferative EPCs had been derived from various pluripotent stem cells such as embryonic stem cells or induced

pluripotent stem cells (Rufaihah et al. 2007, 2010; Pasha et al. 2011; Clayton et al. 2018). On the same note, angiomyogenic repair has also been reported by MSCs-derived induced pluripotent stem cells (Buccini et al. 2012). So far, EPCs can be developed from individual's reprogrammed fibroblasts, and this procedure does not require induction of pluripotency, thus lowering the teratogenicity risk.

Moreover, there are novel opportunities to stimulate resident EPCs to undergo (trans)-differentiation and proliferation by exogenously delivered extracellular vesicles loaded with appropriate active molecules and noncoding RNAs. Overall, the concept of support of vascular reparation through engrafting EPC appears to be promising (Zhang et al. 2012). The chapter provides an in-depth discussion on the previously published evidence regarding the mechanisms of regulation of vascular repair by resident regenerative vascular cells and results of preclinical and clinical studies for vascular functional recovery and reparation using EPCs.

Endothelial Progenitor Cells: Definition and Biological Function

EPCs are defined as immature primitive cells that originate from hematopoietic stem cell precursors and have a thoroughly high ability to differentiate into mature endothelial cells (Asahara et al. 1999). The bone marrow is the primary source of EPCs from which they extravasate into peripheral circulation and then mobilize to be recruited to the foci of pathophysiological processes, such as reparation, vasculogenesis, protection against ischemia/hypoxia, and cell proliferation in response to the chemical cues emanating from injured tissue (Kawamoto and Asahara 2007; Au et al. 2008; King and McDermott 2014). The experimental animal model–based preclinical studies and clinical studies have shown that, being a source for mature cells, these cells have been incorporated into active cores of neovascularization, thus demonstrating their impressive capability to attenuate angiogenesis and vascular function in vivo (Yoder 2012; Jalilian et al. 2020). Therefore, the secretome of EPCs contains a broad spectrum of active molecules, including E-selectin and P-selectin, as well as peptides and growth factors (vascular endothelial growth factor [VEGF], fibroblast growth factor [FGF]-1 and FGF-2, granulocyte-macrophage colony-stimulating factor, angiopoietin-1, von Willebrand factor (vWF), RANTES, brain-derived neurotrophic factor [BDNF], hepatocyte growth factor [HGF], insulin-like growth factor (IGF), glial cell–derived neurotrophic factor [GDNF], nerve growth factor [NGF], placental growth factor [PLGF], and neurotrophin-3 [NT3]), which are involved in the modulation of angiogenesis and improve vascular integrity and function (Duda et al. 2007; Barile et al. 2017). The molecular mechanisms of EPC-induced tissue reparation have been summarized in Fig. 1.

EPCs express many surface antigens, which are common for hematopoietic stem cells (CD34, CD117, CD133, Flk-1, and Flt-4), endothelial cells (CD31, CD62e, CD105, CD106, CD146, and CD309), and mononuclear cells (Tie2+), but lack in the expression of hematopoietic cell-specific marker (CD45) (Mihail et al. 2003). Therefore, some populations of EPCs may lose CD133 antigen within differentiation

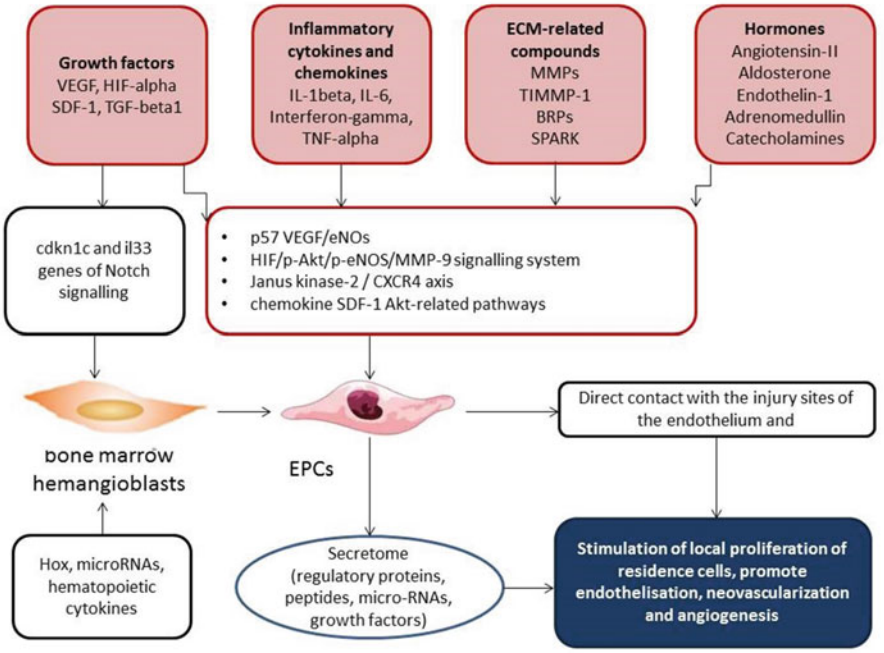

Fig. 1 Underlying molecular mechanisms of favorable effects of EPCs on tissue reparation and vasculogenesis. Abbreviations: EPCs, endothelial progenitor cells; RNA, ribonucleic acid; HIF, hypoxia-inducing factor; NO, nitric oxide; eNOS, endothelial NO synthase; SDF, stromal cell-derived factor-1; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; CXCR4, C-X-C chemokine receptor type 4; Akt1, RAC-alpha serine/threonine-protein kinase; Hox, transcriptional modulator of cell-cell and cell-extracellular matrix adhesionBesides, CD34+ late outgrowth EPCs have been yielded to be reproduced tubes and colony shaping in response to angiogenic growth factors and had higher proliferative potency when compared with CD34-negative late outgrowth EPCs (Hristov 2003)

period and may express on their surface other antigens, i.e., CD144, endothelial NO synthase (eNOs), and vWF.

EPCs expressed sufficient distinguish in self-renewal ability that is determining by colony-forming technique. Depending on ability to appear in fibronectin-coated dish EPCs have been divided onto early outgrowth EPCs (5–7 days after fibronectin plating) or late outgrowth EPCs (7–10 days after fibronectin plating) (Reskiawan et al. 2020).

Finally, EPCs, which originate from the bone marrow hemangioblasts, are released into peripheral blood and involved in vascular repair (Miller-Kasprzak and Jagodziński 2007; Duan et al. 2006). Therefore, some CD34 + CD45- and CD133 + CD34 + CD45- subsets of EPCs may originate from circulating monocytes (Lin et al. 2000; Timmermans et al. 2007). Yet, there are resident EPCs housing in an area between the medial and adventitia layer of the vasculature. They predominantly contain CD34+, but CD31-, which also express VEGFR2, VE-cadherin,

CEACAM1, and TIE2. Besides, they are clonogenic and show proliferative potency that is similar to bone marrow–derived EPCs (Zengin 2006). Thus, EPCs constitute a heterogeneous population of primitive cells with different proliferative activity and angiopgenic potential. They are represented as tissue-resident and circulating populations, and serve as an endogenous source of stem/progenitor cells for postnatal angiogenesis and reparation (Ergün et al. 2007). In addition, the vasa vasorum in the vessel wall plays a pivotal role in transfer EPCs to the place of injury in the intimal region (Table 1).

Table 1 Molecular maker antigens for EPC populations

Antigen name	Alternative name	Distribution	Biological function
CD31	Endocam, PECAM-1	Monocytes, platelets, granulocytes, lymphocytes subsets	Cell adhesion, signal transduction, receptor for CD38
CD117	c-kit, SCFR	Hematopoetic progenitor cells, mast cells	SCF receptor, regulator of differentiation/proliferation of hematopoetic progenitor cells
CD34	MY10, mucosialin	ECs, embryonic fibroblasts, hematopoetic precursors	Stem cell marker, adhesion, receptor for CD62 ligand
CD105	Endoglin	ECs, bone marrow subsets, activated macrophages	Cellular response to TGF-beta1, adhesion, embryonic angiogenesis
CD106	VCAM-1	ECs	Adhesion of immune cells to the endothelium during inflammation
CD133	AC133, prominin-like 1	HSCs subsets, ECs, epithelial cells	Unknown
CD144	VE-cadherin, cadherin-5	ECs, stem cells	Adhesion, endothelium integrity, and biology
CD146	MUC18, S-endo	ECs, melanomas	Adhesion, target of CD44 signaling
CD309	KDR, VEGFR2	ECs, angiogenic precursors, hemangioblasts	Binding VEGF, regulation of cell adhesion and signaling
vWf	–	ECs	Antihemophilic factor, platelet-vessel wall mediator of coagulation
CD62E	E-selectin	ECs	Initial attachment of circulating leukocytes to the endothelium during inflammation
Tie-2	Tek	Embryonic and adult ECs, HSCs, circulating proangiogenic monocytes	Transmembrane receptor tyrosine kinase

Abbreviations: EPCs, endothelial progenitor cells; CD, cluster of differentiation; HSCs, hematopoietic stem cells; VEGFR, vascular endothelial growth factor receptor; MUC, melanoma cellular adhesion molecule; vWf, von Willebrand Factor; SCFR, mast/stem cell growth factor receptor; PECAM, platelet/endothelial cell adhesion molecule; TGF-beta1, transforming growth factor beta 1; VCAM-1, vascular cell adhesion molecule-1

Endothelial Progenitor Cell Dysfunction in Cardiovascular Diseases

Numerous preclinical studies in the experimental animal models and clinical studies have unveiled strong relations between a decreased number, weak function, and lowered survival of EPCs in the peripheral blood with a high risk of cardiovascular diseases and cardiovascular events (Akhavani et al. 2007; Ahmed et al. 2016; Berezin et al. 2016a; Sen et al. 2011; Montenegro et al. 2018; Pelliccia et al. 2009a; Sata 2006; Rahmawati et al. 2015). This relationship has been attributed to the EPCs' dysfunction, and it has been found to serve as a powerful predictor for the impaired reparability (Peng et al. 2015; Berezin, 2016). Moreover, overt cardiovascular diseases, such as stable coronary artery disease, atherosclerosis, HF, chronic kidney disease, metabolic diseases including diabetes mellitus, abdominal obesity, and metabolic syndrome, are associated with the development of EPCs dysfunction (Kiewisz et al. 2016; Siebel et al. 2010; Psaltis and Simari 2015; Berezin et al. 2016b; Kachamakova-Trojanowska et al. 2015). Besides, insulin resistance, nicotinamide adenine dinucleotide phosphate oxidase–mediated oxidative stress, inflammatory cytokines, Akt/PI3K, and sirtuin 1-related micro-RNAs can negatively modulate EPCs' biological characteristics and functional activities, such as extravasation, mobilization, migration, and participation in neovascularization and angiogenesis (Li et al. 2018; Ait-Aissa et al. 2020; Berezin and Berezin 2019). However, it remains unclear whether altered vascular reparation due to EPCs' dysfunction comes before the onset of cardiovascular disease or vice versa that it is the outcome of conventional cardiovascular risk factors on transcriptional potency of these precursors (Ozkok and Yildiz 2018; Psaltis et al. 2011; Xu et al. 2012).

Some studies have shown that the dysfunction of EPCs occurs over a certain period after contact with CV risk factors (Lavoie and Stewart 2012). It has also been reported that acute cardiac and vascular events were associated with a reasonably early increase in the number of EPCs in circulation and consequent decline in their number over time, but the functionality of EPCs was frequently impaired before the onset of cardiovascular events, and it was not closely related to the number of circulating EPCs and variability/total number of cardiovascular risk factors (Altabas et al. 2016; Regueiro et al. 2015). Perhaps these controversial results reflect preemptive adaptation of the endogenous repair system to the epigenetic stimuli corresponding to cardiac and vascular remodeling (Minhajat et al. 2015; Pelliccia et al. 2009b). Indeed, a preexisting reduced number of EPCs might correspond to previous severe conditions, including trauma infections born preterm but not merely cardiovascular diseases and cardiovascular risk factors (Bertagnolli et al. 2018; Roth et al. 2019). For instance, a systematic review and meta-analysis of six clinical studies ($n = 236$) have shown that exercise training leads to an increase in the number of circulating EPCs in patients with overt CV disease (Cavalcante et al. 2019). Finally, the abrogation of EPCs' differentiation potential to become mature endothelial cells and completely restore vascular integrity and function that persists for a long time alleviates target organs' damage (Pyšná et al. 2019).

EPCs' severe dysfunction is generally considered as an indicator of poor prognosis, while restoration of the number and function of EPCs are viewed as a

biomarker of a consequent improvement of clinical outcomes (Wils et al. 2017; Mandraffino and Saitta 2018). The analysis of the results of 34 studies has shown that a reduced baseline EPCs' level was associated with a significantly increased risk of cardiovascular events all-cause mortality onset and progression of micro-angiopathy (Rigato and Fadini 2018). Among patients with HFrEF/ HFpEF, a reduced number and/or weak function of circulating EPCs were strong predictors of cardiovascular events and HF-related outcomes (Berezin et al. 2016). Overall, EPCs' dysfunction predicts cardiovascular events independent of traditional and nontraditional CV risk factors (Bakogiannis et al. 2012).

Early Outgrowth and Endothelial Colony-Forming Progenitor Cells in Revascularization and Vascular Reparation

The number of circulating EPCs in peripheral blood is extremely low and their adequate isolation is generally aimed at their therapeutic application for vascular reparation. This necessitates their ex vivo expansion and manufacturing to generate early and late outgrowth EPCs. Although both phenotypes of EPCs have demonstrated regenerative ability, functional analyses of these cells have shown that late outgrowth EPCs were superior to early outgrowth EPCs in their tubulogenic potential on matrigel maintenance of endothelial cell, and angiogenesis. Preclinical experimental applications of the early and late EPCs in several animal models have been listed in Table 2.

Animal Studies for Vascular Repair with EPCs

There is a large body of evidence in the published literature about the infusion of EPCs of hematopoietic origin to treat ischemic limbs in an immunocompromised mice experimental animal model. The results showed substantial improvement in terms of vascular perfusion and tissue recovery from ischemic injury (Crosby et al. 2000; Göthert et al. 2004; Peters et al. 2005; Zentilin et al. 2006). Granulation tissue formation and neovascularization can be induced by nonhematopoietic EPCs from the spleen. Li et al. (2017) reported that tail vein injection of EPCs pretreated with VEGF were able to accelerate the re-endothelialization and inhibit neointimal formation through connexion-43-mediated mechanisms in an experimental mice model.

Experimental animal studies have shown that systemic infusion of EPCs derived from the perivascular niche in the liver were able to enhance angiogenesis and attenuate blood perfusion defects in ischemic hind limbs (Murasawa and Asahara 2005). Moreover, it has been reported that non-bone-marrow-derived c-kit+CD45-34+ EPCs contributed to the postnatal vascular reparation akin to the bone marrow–derived EPCs (Aicher et al. 2007). These studies concluded that the early growth EPCs promoted vascular reparation and cardiac regeneration through paracrine mechanism.

Table 2 Preclinical applications of early and late EPCs

Animal model	Cell source	Route	Effects	References
Early EPCs				
Ischemic stroke	PB	LI	↑ vascular perfusion ↑ recovery of ischemia-induced tissue injury	Crosby et al. (2000), Göthert et al. (2004), Peters et al. (2005), Zentilin et al. (2006)
Limb ischemia	Spleen	SI	↑ endothelialization ↑ granulation tissue formation ↓ neointimal formation	Li et al. (2017)
Limb ischemia	PB	SI	↑ angiogenesis and blood perfusion in ischemic hind limbs	Murasawa and Asahara (2005)
Late EPCs				
Ischemic stroke	hUCB	LI	↑ functional restoration ↑ angiogenesis ↑ neurogenesis ↓ apoptosis	Ding et al. (2016)
Ischemic stroke	UCB	LI	↑ reperfusion ↑ regenerative effect on BBB disruption ↓ cerebral apoptosis ↑ CBF	Garrigue et al. (2016)
Ischemic stroke	UCB	LI	↑ neurological functional recovery ↑ angiogenesis ↑ neurogenesis	Moubarik et al. (2011)
Ischemic stroke	PB	LI	↑ structure and function of BBB	Abdulkadir et al. (2020)
Traumatic brain injury	hUCB	LI	↓ neurologic disability ↑ microvessel density ↑ expression of the proangiogenic growth factors	Zhang et al. (2013), Huang et al. (2013)
Ischemic retinopathy	hUCB	LI	repairing the retinal vasculature and ↓ ischemic injury	Bertelli et al. (2020), Park et al. (2014)
Ischemic Retinopathy	hUCB	LI	↑ reparative angiogenesis in the ischemic retina	O'Leary et al. (2019)
Oxygen-induced retinopathy	hUCB	LI	↓ developing retinal vasculature	Li Calzi et al. (2019)
Limb ischemia	hUCB	LI	↑ muscle perfusion	Kang et al. 2017
Limb ischemia	PB	i.v.	↑ capillary collateral formation in ischemic leg	Minami et al. (2015)
Acute kidney injury	hUCB	LI	↑ kidney perfusion ↓ occurrence of apoptosis	Viñas et al. (2016)
Kawasaki disease	PB	i.v.	↑ coronary artery wall integrity ↓ occurrence of vascular aneurysm	Chen et al. (2012)

(continued)

Table 2 (continued)

Animal model	Cell source	Route	Effects	References
MI	PB	IMI	thymosin-β4-pretreated EPCs influenced ↑ capillary density and LVEF	Poh et al. (2020)

Abbreviations: ↑ increase; ↓ decrease; BBB, blood-brain barrier; CBF, cerebral blood flow; EPCs, endothelial progenitor cells; hUCB, human umbilical cord blood; IMI, intramyocardial injection; i.v., intravenous injection; LVEF, left ventricular ejection fraction; LI, local injection; MI, myocardial infarction; PB, peripheral blood; SI, systemic infusion; UCB, umbilical cord blood

Although there are limiting data that early outgrowth EPCs are more effective to produce significantly higher levels of proangiogenic factors such as VEGF-A, VEGF-B, SDF-1, and insulin-like growth factor (IGF)-1 when compared with late outgrowth EPCs (Urbich et al. 2005), a large majority of the investigators have suggested that the blood-derived late outgrowth EPCs could be formidable paracrine mediators besides being potent regulators of the regenerative potential of the tissue-resident stem cells via PDGF-BB/PDGFR-β signaling (Lin et al. 2014). The late outgrowth EPCs, also known as endothelial colony-forming progenitor cells (ECFCs), are promising candidates for cell-based therapies as they are rich in EGF, HGF, VEGF-A, PLT-derived growth factor-B, interleukin-8, and monocyte chemoattractant protein-1, all featuring the high potential for promoting postnatal proangiogenic activity in vivo and lack telomerase activity (Tasev et al. 2015; Banno and Yoder 2018).

In an experimental animal photothrombotic model of ischemic stroke in mice, ECFCs derived from human umbilical cord blood were infused in the ischemic brain tissue that led to functional restoration, improved angiogenesis, neurogenesis, and substantially decreased apoptosis in the ischemic area (Ding et al. 2016). Additionally, in rats with experimentally induced ischemic stroke, local implantation of ECFCs, pretreated with erythropoietin, significantly promoted their homing-in capacity to the ischemic site after transient middle cerebral artery occlusion followed by reperfusion. Incidentally, cell therapy also erythropoietin treatment potentiated their protective capability and regenerative effect of ECFCs on the blood-brain barrier disruption through attenuated apoptosis and supporting improved cerebral perfusion (Garrigue et al. 2016). ECFCs with a greater proliferative and directional migratory capacity effectively stimulated the differentiation of the endothelial precursors to rejuvenate blood-brain barrier (Abdulkadir et al. 2020). In an experimental animal model of transient middle cerebral artery occlusion, local injection of ECFCs significantly improved the functional recovery besides significant regression of the neurological deficiency (Moubarik et al. 2011). On the same note, local injection of ECFCs was more effective in promoting neovascularization and restoring blood-brain barrier integrity in an experimental model of traumatic brain injury. The cell-based therapy approach also successfully alleviated neurologic deficiency by increasing the brain microvessel density and expression of the proangiogenic growth factors such as SCD-1 and VEGF (Zhang et al. 2013; Huang et al. 2013).

The ECFCs-based treatment effectively repaired the retinal vasculature and attenuated ischemic injury of the retina in a rodent experimental model of ischemic retinopathy (Bertelli et al. 2020; Park et al. 2014; Medina et al. 2010). Moreover, it was found that intravitreal delivery of ECFCs were successfully incorporated into the damaged retinal vasculature as part of the repair process and sufficiently reduced the avascular area (O'Leary et al. 2019). Moreover, this effect has been mediated by the transfer of extracellular vesicles containing multiple-microRNAs hyaluronic acid receptor and insulin-like growth factor–binding proteins (Dellett et al. 2017; Sakimoto et al. 2017). Using a mouse model of oxygen-induced retinopathy, Li Calzi et al. established that human progenitor cell combination of bone marrow–derived CD34+ EPCs and vascular wall–derived ECFCs synergistically contributed to the protection and development of retinal vasculature against injury (Li Calzi et al. 2019). In addition, ECFCs were able to protect against retinal degeneration by suppressing matrix metalloproteinase activation, attenuating inflammatory signaling, protecting vascular smooth muscle cells from apoptosis, and mediating the effects of endothelial nitric oxide.

There is strong evidence that the administration of human cord blood ECFCs or their exosomes protected mice against renal ischemia/reperfusion injury via transfer of micro-RNA-486-5p that targets the phosphatase and tensin homolog and the activation of the Akt pathway (Viñas et al. 2016).

Intravenous engraftment of late outgrowth EPCs derived from human peripheral blood mononuclear cells into immunocompromised mice after experimentally induced unilateral hind limb ischemia revealed higher angiogenic potential than classically defined early outgrowth EPCs (Minami et al. 2013). Treatment with ECFCs in combination with mesenchymal progenitor cells effectively restored blood flow in the ischemic skeletal muscle in an experimental murine model of hind limb ischemia (Kang et al. 2017).

Chen et al. reported that intravenous injection of bone marrow–derived, in vitro expanded EPCs in mice with the model of Kawasaki disease was associated with the accelerated repair of coronary artery endothelial lesion and decreased the occurrence of vascular aneurysm for 56 days (Chen et al. 2012). Investigators also observed that the number of peripheral EPCs in the Kawasaki disease model group was significantly lower when compared to the circulating number of EPCs in both transplanted and control groups.

Interestingly, intramyocardial injection of thymosin-β4-pretreated EPCs following acute myocardial infarction significantly increased capillary density and left ventricular pump function in diabetic rats, while naïve (nontreated) EPCs did not exhibit any therapeutic benefits in terms of tissue regeneration due to poor migration, weak tubulogenic potential, and weak proangiogenic paracrine activity (Poh et al. 2020). These data showed that autologous bone marrow–derived EPCs from diabetic rats might have limited regenerative potential and lacked the ability to sustain endothelial integrity and cardiovascular protection due to the effect of hyperglycemia.

Thus, autologous EPCs-based therapy seemed as a personalized therapeutic approach to vascular reparation while these cells received from patients with a

wide range of cardiovascular risk factors and CV diseases can be dysfunctional and require to be specifically repaired prior to use. In this context, ECFCs and their combination with another type of progenitor precursors appear to be more promising in the therapeutic perspective.

Clinical Studies for EPCs Applications

The intriguing scientific data from the experimental animal studies supports the potential clinical applications of EPCs and requires confirmatory proof in the clinical settings before their routine application as a therapeutic option for patient (Bianconi et al. 2018). Nevertheless, numerous clinical studies and large clinical trials have provided inconsistent results for the therapeutic efficacy of human bone-marrow-derived CD34+ EPCs infusion (Itescu et al. 2002). For instance, no therapeutic benefits were observed in terms of improvement in coronary artery perfusion regrowth and myocardial angiogenesis following acute myocardial infarction (Kocher et al. 2001; Iwasaki et al. 2006). During a single-center prospective randomized double-blinded phase I clinical trial, which included 39 patients having end-stage diffuse coronary artery disease unsuitable for percutaneous and surgical coronary revascularization, treatment with CD34+ cells did not show any significant improvement in LVEF in survivors when compared with nonsurvivors for 5-year follow-up (Sung et al. 2018). At the same time, the analysis of angiographic findings showed that the angiogenesis was significantly increased and left ventricular remodeling was sufficiently ameliorated. Besides, the clinical scores for angina and HF were significantly reduced over 5 years of follow-up. In another clinical trial, intravenous CD34+ cell-based therapy was safe and efficacious in improving left ventricular function for patients with severe diffused CAD unsuitable for coronary intervention and poor response to pharmacotherapy (Lee et al. 2015). Patients with refractory angina, who were treated with intramyocardial injections of autologous CD34+ EPCs, experienced significant alleviation in angina frequency and tolerance to physical exercise over 6 months after treatment (Losordo et al. 2011). Henry et al. demonstrated that intramyocardial delivery of autologous CD34+ cells into the ischemic myocardial zone was associated with stable improvement in angina and a sustained trend to decline of major adverse cardiac events including death, myocardial infarction, acute coronary syndrome, or hospitalization due to HF over 24 months (Henry et al. 2016). Finally, in the RENEW (Efficacy and Safety of Targeted Intramyocardial Delivery of Auto CD34+ Stem Cells for Improving Exercise Capacity in Subjects with Refractory Angina) trial, it was observed that autologous CD34(+) cell-based therapy was as safe as placebo treatment, while the study was early terminated by the sponsor for strategic considerations (Povsic et al. 2016).

Overall, three double-blind randomized trials ($n = 304$) compared intramyocardial delivery of autologous CD34+ EPCs with intramyocardial placebo treatment to affect total exercise time, angina frequency, and major adverse cardiac events. Additionally, analyses of the data from the phase I, phase II, ACT-34, ACT-34 extension, and phase III RENEW trials have shown that autologous

CD34+ EPCs therapy significantly decreased mortality and numerically reduced major cardiovascular events during 24 months follow-up (Henry et al. 2018).

Khan et al. reported the results of meta-analysis of six clinical studies for cell-based therapies in refractory angina showed that CD34(+) EPCs transplantation was safe and led to sufficient improvement in angina frequencies, relevant clinical outcomes, and myocardial perfusion (Khan et al. 2016). Another meta-analysis has shown that intramyocardial delivery of CD34+ EPCs was superior to placebo treatment and decreased a risk of all-cause mortality, attenuated angina frequency, and increased exercise time that was without a significant increase in adverse events (Velagapudi et al. 2019). Moreover, it has been observed that CD34+ EPCs-based therapy for refractory angina patients was associated with sufficient improvement in cardiovascular mortality and a reduction in hospital visits and overall total costs for cardiac procedures over 1-year period of follow-up (Johnson et al. 2020). In patients with chronic angina on optimal medical therapy, cell therapy improved clinical symptoms, increased exercise capacity, and left ventricular ejection fraction, but did not show any effect on prognosis (Shah et al. 2018).

In patients with severe nonischemic HFrEF, transendocardial transplantation of CD34+ EPCs in the areas of myocardial hibernation appeared to be effective in improvement of diastolic function (Bervar et al. 2017). Therefore, intracoronary transplantation of CD34+ EPCs positively impacted myocardial perfusion in patients with nonischemic HFrEF due to dilated cardiomyopathy (Lezaic et al. 2015). In addition, improved global left ventricular function, increase in exercise tolerance, and long-term survival were attributed to intracoronary delivery of CD34+ EPCs in nonischemic HFrEF (Vrtovec et al. 2013a). It has been observed that transendocardial transplantation of CD34+ EPCs was associated with more pronounced myocardial cell retention rates, thus leading to greater improvement in left ventricular systolic function as compared to intracoronary route. However, the circulating levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) and 6-minute walk distance were comparable in patients from the two groups of patients (Vrtovec et al. 2013b). Interestingly, repeated CD34+ EPCs administration offered no additional benefits in alleviating the clinical course of the disease, exercise tolerance, left ventricular pump function, or the circulating NT-proBNP levels when compared with single-dose cell therapy (Vrtovec et al. 2018). Patients with ischemia-induced HFrEF demonstrated dose-dependent effect of CD34+ EPCs transplantation on improvement of cardiac pump function, exercise tolerance, and circulating levels of NT-proBNP (Poglajen et al. 2014).

Despite the encouraging data from experimental animal studies as well as from some of the reported clinical studies, CD34+ EPCs delivery has been also disputed as a promising strategy to induce therapeutic angiogenesis in patients with critical limb ischemia (Lara-Hernandez et al. 2010). The prospective randomized, double-blinded, placebo controlled, multicenter study (RESTORE-CLI) has shown that intramuscular injection of autologous bone marrow–derived EPCs was safe and associated with alleviation of the disease progression when compared to placebo treatment (Powell et al. 2011). However, meta-analysis of placebo-controlled trials showed no benefit from bone marrow–derived cell therapy on the primary clinical

outcome (amputation, survival, and amputation-free survival) in patients with critical limb ischemia (Peeters Weem et al. 2015).

To sum up, the reparative potential of the cell therapy strongly depends on a wide range of factors, including phenotype of the cells, cell dose, delivery route, and timing. Pretreatment of EPCs (predominantly with bone morphogenetic protein 4, erythropoietin, osteoprotegerin [fucoidan], or their delivery in combination with other progenitor types such as mesenchymal stem cells, epigenetic reprogramming, and genetic manipulation prior to transplantation) is crucial for improved prognosis and successful outcome of therapeutic angiogenesis (Faris et al. 2020).

Conclusion and Future Prospects

It has been postulated that culture-expanded EPCs, which express CD31 and CD14, or autologous EPCs are alternative for the cells obtained from human umbilical cord tissue for transplantation (Muniswami et al. 2020; Kraus et al. 2021). Similarly, autologous circulating mononuclear cells can be a source for pre-programmed cells to obtain EPCs with required phenotype (Siegel et al. 2018). Their isolation and manufacturing may correspond to current technologies according to good manufacturing practice (Faris et al. 2020). Further development in cell processing technology for efficient isolation, expansion, and transplantation, or mobilization and recruitment of EPCs into target tissues are expected to be investigated and stablished in translational studies and in large clinical trials in the near future.

In conclusion, therapy with early and late outgrowth EPCs is promising strategy for vascular reparation while a vision of future prospects of the approach closely relates to pretreatment of the cells with epigenetic reprogramming and genetic manipulation. Perhaps, high variability of clinical results of the cell therapy is a result of uncertain functional heterogeneity of EPCs implanted due to several conventional and not yet fully established risk factors.

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Neural Stem Cells

27

From Bench to Bedside

Yash Parekh, Ekta Dagar, Khawaja Husnain Haider, and Kiran Kumar Bokara

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Abstract

The introduction of neural stem cells (NSCs) from adult mouse brains by Brent Reynold and Samuel Swiss in 1992 not only paved the way for in vitro culturing but also opened the way for understanding neurophysiology and disease pathology with better insights into neurobiology. In this regard, in recent years, most of the attention has been raised by the stimulating prospects of NSC application for cell replacement therapies for neurological disorders. Despite the evident benefits pledged by NSC application, which showed encouraging neuroregeneration events in preliminary studies on animal models, there remains a gap between theory and practice for clinical use. Therapies using stem cells were successful for the treatment of epidermal and corneal disorders. However, applications for

Y. Parekh · E. Dagar · K. K. Bokara (✉)
CSIR–Centre for Cellular and Molecular Biology, Hyderabad, India
e-mail: yashp@ccmb.res.in; ektadagar@ccmb.res.in; bokarakiran@ccmb.res.in

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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diseases affecting the nervous system remain a pioneering field and are still in their infancy. With the increasing demand for therapeutics and the clinical prevalence of neurodegenerative diseases, an understanding of the basic biology of NSCs, the niche/microenvironment governing stem cell characteristics, and the methods for manipulating these cells could provide reliable, safe, and effective outcomes in cell replacement approaches, which could be beneficial for the clinical use of NSCs.

Keywords

3D culture · Central nervous system · ESCs · iPSCs · Neurogenesis · Neuron · Neuronal stem cells · Organoids · Stem cells

Abbreviations

BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
bHLH	Basic-helix-loop-helix
BMP	Bone morphogenetic proteins
CNS	Central nervous system
CRABP1-2	Cellular-RA-binding proteins
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
hNPCs	Human neural progenitor cells
iPSCs	Induced pluripotent stem cells
IVF	In vitro fertilization
MSCs	Mesenchymal stem cells
NPCs	Neuronal progenitor cells
NSCs	Neural stem cells
PD	Parkinson's disease
PTC1	Patched
RA	Retinoic acid
RALDH1–3	Retinaldehyde dehydrogenases 1, 2, and 3
RAR-1–3	RA receptors
RGC	Radial glial cells
RXR1–3	Retinoic X receptors
Smo	Smoothened
SOX	SRY-related HMG box
SVZ	Subventricular zone
TGF-beta	Transforming growth factor-beta
TLE	Temporal lobe epilepsy

Introduction

Until the 1990s, it was believed that the generation of new neurons does not occur in the fully developed adult mammalian brain. On the contrary, stem cells are the key players involved in developing and maintaining the tissues in the mammalian system, giving rise to new cells and replenishing dead cells or worn-out cells. New techniques have emerged to successfully demonstrate that new neurons are continuously generated in the adult brain, a concept that is contrary to the long-standing dogma that neurons are terminally differentiated cells and do not proliferate during postnatal life.

With the increasing literature and experimental evidence from neuroscientists, it is now believed that in a mammalian brain, neural stem cells (NSCs) give rise to brain cell types, regardless of the age of the animal, except that their proliferative potential declines with advancing age. With the advent of new technologies and methods, it is now possible to isolate and culture neural stem cells in vitro from central nervous system (CNS) tissues with neuron-specific growth factors, generating in vivo tissue-like structures. The in vitro neuronal culture methods pave the way for understanding neurogenesis and neuropathogenesis, thereby facilitating the development of both in vitro and in vivo models for research and their subsequent use in therapeutic applications.

Induced pluripotent stem cells (iPSCs) developed by the reprogramming of somatic cells (Takahashi and Yamanaka 2006) have paved the way for the generation of patient-specific stem cells, which has given genuine hope for the practice of personalized medicine for both adult and pediatric applications (Cagavi et al. 2018; Çetinkaya and Haider 2021). The iPSC technology has enabled researchers worldwide to generate three-dimensional (3D)/organoid cultures with different cell types, including cerebral organoids (Yan et al. 2020; Logan et al. 2020). Unlike other techniques, the 3D/organoid culture technique has its relevant challenges and limitations; however, it is still considered the most preferred technique, which can give rise to organ-like features under in vitro culture conditions and mimic the natural habitat of cells in vivo (Artegiani and Clevers 2018). Considering the complexity of organs, the present organ-on-chip and assembloid culturing techniques would enable the development of organ systems (Wu et al. 2020).

In this chapter, we discuss the origin and progression of neuroscience and the challenges associated with it. In the later part, we have addressed the current advancements in the techniques used in research, the role of the microenvironment in influencing stem cell characteristics, therapeutic applications and the challenges associated with them, and the ways to overcome those challenges.

Historic Prospective of Neural Stem Cells

Pablo Garcia-Lopez et al (2010) documented Cajal's contribution towards understanding the basic concepts of vertebrate neural system. His work suggested various theories, including “neuron doctrine,” law of dynamics, neurogenesis, functional polarization of electrical potential, neural plasticity, neuronal

regeneration, and degeneration. These have led ways for modern neuroscience, contributing immensely to the expansion of the field. Santiago Ramon y Cajal and Camillo Golgi (Swanson and Lichtman 2016) laid the foundation for modern neurosciences by investigating the intricacies of macro- and micro-structures in the human nervous system. He also stated that "once the development was completed the adult centres, the nerve paths were something fixed and immutable. Everything may die, nothing may be regenerated." This statement continued to remain the central dogma until the 1960s, when neural stem cells were discovered.

The conviction of numbness of neurogenesis in the adult human brain was based on the following four major points:

1) Clinical evidence showing that patients suffering from neurological diseases/disorders do not show any signs of recovery or reduction in symptoms – these observations made the researchers believe that neurogenesis does not occur in the human adult brain.
2) The function of neuronal cells, which consists of complex networks and requires precise communication as a slight change in the system can alter the behavior and functions of an organism.
3) During learning and memory processes, memory is the case of recalling something, which already stored with the stable neuronal connections, and new neurons were seen as unwanted and incompetent with the memory processes.
4) Lack of techniques and resources needed to understand neurogenesis and its associated mechanisms.

In the 1960s, initial reports from Joseph Altman (1962) and his colleagues suggested that cells in the dentate gyrus and hippocampal region can incorporate radioactive thymidine, which can only be possible in the case of dividing cells and can be visualized using autoradiography. Limited by the availability of specific molecular markers, Altman and colleagues could not prove them as neurons; the data emanating from their experiment suggested that the adult human brain had dividing cells, whether stem cells or progenitor cells. Later in the 1970s, Michel Kaplan (Kaplan and Hinds 1977) used a combination of autoradiography and electron microscopy to observe the dividing neurons. In the 1980s, Nottebohm and collaborators reported that neurogenesis occurred in the adult brains of birds, suggesting the neurological basis of songs in birds. They used BrdU and neural molecular probes, with confocal microscopy (Nottebohm 2002). During the 1990s, researchers successfully isolated and propagated neural stem cells from the adult mammalian brain in vitro, using specific growth factors and morphogens.

During the twentieth century, several research groups developed techniques to culture and maintain the nervous tissue in vitro (Pacitti et al. 2019). In the year 1907, a report suggested culturing CNS and medullary tubes from frog embryos (Harrison 1907). Over the years, scientists tried various culture conditions for culturing cells derived from the different parts of mammalian CNS. In 1964, Goldstein and colleagues published a study reporting successful the in vitro culturing of an immortalized cell line from a neuroblastoma cancer patient (Goldstein et al. 1964). In 1989, Knopfel reported successfully isolating NSCs from rat forebrain, thus opening a new

horizon of infinite possibilities and breaking all barriers that hinder research and clinical approaches for neuronal development and modeling various neuropathological conditions (Knöpfel et al. 1989). In 1992, Reynolds and Weiss reported the 3D culturing of NSCs from murine sources and differentiated them into neurons and astrocytes, using a cocktail of specific growth factors (Reynolds and Weiss 1992). During the twenty-first century, various protocols were explored extensively for the isolation of embryonic stem cells (ESCs) and the generation of human-induced pluripotent stem cells (hiPSCs) (Khan et al. 2018; Castro-Viñuelas et al. 2020). The directed and unguided differentiation protocol gave rise to new model systems, known as organoids, providing more insights into the CNS system in vitro and allowing us to understand the brain from an evolutionary perspective (Chiaradia and Lancaster 2020). Several reports have suggested modeling neurological disorders using organoid-based culture techniques. With the increasing requirement to understand the complexity and cross talk between various organ systems, the organ-on-chip technique was developed, which involves coculturing cells from different origins, creating a more complex model system, and mimicking more tissue-like natural conditions. In this line, brain-on-chip technology, along with blood-brain barrier (BBB) was developed, which mimic in vivo brain-like conditions (Bang et al. 2019; Maoz 2021). This model could enable us to understand the developmental process and eventually BBB modeling.

Neural Stem Cell Culture – Its Applications and Limitations

In 1989, Sally Temple suggested a protocol to culture NSCs from the septal tissue of E13.5–E14.5 rat brain (Temple 1989). Later, Reynolds and Weiss cultured NSCs from an adult rodent brain in a serum-free medium (Reynolds and Weiss 1992). The report suggested that the presence of epidermal growth factor (EGF) without any adhesive factor is required for maintaining NSC proliferation. Upon providing the attachment factor, i.e., poly-L-ornithine, the cells would stop proliferating and start to differentiate even in the presence of EGF. The differentiation would not give rise to multineuronal types, which would require a combination of different growth factors (Reynolds and Weiss 1992). Later on, it was found that as compared to brain-derived NSCs, spinal-cord-derived NSCs showed better growth kinetics in the presence of EGF and basic fibroblast growth factor (bFGF) (Kornblum 2007). Irvin et al. suggested that the use of EGF alone resulted in the higher commitment of the cells toward glial cell lineage, whereas the use of bFGF preferentially promoted neurogenesis (Irvin et al. 2003).

The culture substrate is one of the most critical determinants of cellular differentiation, and it also defines the fate of the cell's lineage. The extracellular matrix (ECM) consists of a complex mix of bioactive molecules and substrate factors required by the cells for attachment. An optimum combination of these components is necessary for the generation of desirable and reproducible in-vivo-like conditions. Hydrogel scaffolds, such as Matrigel and/or brain-tissue-derived ECM hydrogel, are widely used for conditions conducive for differentiation, besides 3D culturing techniques. These

hydrogels facilitate the slow and controlled release of growth factors, which helps in the regulated growth and development of cells. Neurosphere culturing methods in two-dimensional (2D) culture conditions has provided greater insight into the dynamics and functioning of neural cells. However, 2D culturing approaches has limitation in mimicking the 3D complexity, as observed in neural tissue. The 3D culture system overcomes the limitations of 2D culture, providing complex organ-like features and allowing better cell-cell interaction (Kapałczyńska et al. 2018).

Lancaster and Knoblich introduced the concept of a whole-brain organoid, representing different brain regions with the same 3D tissue (Lancaster and Knoblich 2014). With the introduction of the organoid technique, several groups have developed various disease models (Dutta et al. 2017; Kim et al. 2020). The use of organoids has facilitated in understanding multiple evolutionary aspects of the human brain as well as other mammalian species. Mattei and colleagues have demonstrated the effect of microgravity on neurogenesis using cerebral organoids placed in the rotatory cell culture system, suggesting that microgravitational changes influence the change in expression in rostral-caudal patterning genes and cortical markers (Mattei et al. 2018) (Table 1). Exogenous sources of neural cells involve the use of the following (Table 2):

1) Fetal and adult rodent NSCs
2) Fluorescence-assisted sorted enriched cells from rodent NSCs
3) Fetal or adult human NSCs based on the availability of abortus or biopsied brain tissue
4) ESC-derived NSCs

To mimic the complexity and variability of the naive brain tissue, various culture techniques have been introduced to replicate better in vivo development and cytoarchitecture.

The iPSC technology, pioneered by Takahashi and Yamanaka (2006), allows generating specific cell types from reprogrammed pluripotent stem cells. The approach involves the reprogramming of somatic cells to pluripotency by the transduction of a quartet of transcription factors, i.e., Oct3/4, Sox2, c-Myc, and Klf4 (Ibrahim et al. 2016). They are considered surrogate ESCs, without any moral or ethical issues relevant to their generation and use. They provide a renewable source of cells and, hence, have drawn the interest of researchers worldwide for theranostic applications. They are also being used to generate models that can closely mimic the human brain and as a means to "replace, reduce and refine" the use of animal models.

The researchers are showing immense interest in the use of NSCs for therapeutic applications and disease modeling in neurosciences. NSC transplantation has been performed for traumatic brain injury, neurodegenerative diseases, and also a few neurological disorders (Yamasaki et al. 2007; Ziaee et al. 2017; Hosseini et al. 2018a; Hosseini et al. 2018b; De Los Angeles 2019). Initially, the isolation of Naive NSCs from CNS sources was practiced to culture and transplant them for therapeutic purposes; however, the proliferative potential of NSCs was compromised during transplantation due to a pathological and cytokine-rich microenvironment in the diseased tissue, further aggravated by infiltrating inflammatory cells. The genetic manipulation of NSCs using

Table 1 Table showing the development of culture strategies of neural cell culture (Pacitti et al. 2019)

Year	Reports	Authors
1907	Fist CNS (medullary tube) culture from frog embryo	Harrison (1907, 1910)
1924	Chick-embryo-explant-based CNS model	Hoadley (1924)
1936	Maintenance of chick embryo head	Waddington and Cohen (1936)
1946	Long-term CNS culture using rolling tubes	Hogue (1946)
1951	Culture-maintained fragments of cerebral and cerebellar cortex	Costero and Pomerat (1951)
1961	Development of modern cell aggregate culture technique	Bousquet and Meunier (Pacitti et al. 2019)
1966	Embryonic rat spinal cord and ganglia explants cultured on collagen glass	Crain and Peterson (1967)
1973–1997	Organotypic cultures from various cerebral regions	LaVail and Wolf (1973), Whetsell et al. (1981), Knöpfel et al. (1989), Østergaard et al. (1995), and Robertson et al. (1997)
1964	First immortalized neuronal line from children with neuroblastoma cancer	Goldstein et al. (1964)
1973	SK-N-SH and SH-SYSY neuroblastoma lines from metastatic bone tumor	Biedler et al. (1973)
1976	PC12: rat-derived adrenal pheochromocytoma line	Greene and Tischler (1976)
1984	Neuroblastoma cells exposed to retinoic acid display neuroblast-like phenotype	Påhlman et al. (1984)
1986	Primary microglia lines from neonatal rat cerebral tissue	Giulian and Baker (1986)
1988	Primary hippocampal neurons from fetal rats	Dotti (Dotti et al. 1988)
1989	First neural stem cells isolated from rat forebrains	Temple (1989)
1990	Primary forebrain neurons from adult canaries	Goldman (1990)
1992	NSCs from adult murine striata and differentiation into neurons and astrocytes	Reynolds and Weiss (1992)
1993	NT2: human neuronally committed teratoma-derived line	Pleasure et al. (1992)
1995	Cortical, hippocampal, cerebellar, and midbrain neurons from rat embryos	Brewer (1995)
1999	Midbrain neurons from rat embryos	Lingor (Lingor et al. 1999)
	First human multipotent NSCs derived from a 10.5-week embryonic diencephalon	Vescovi et al. (1999)

(continued)

Table 1 (continued)

Year	Reports	Authors
2005	Secondary immortalized mouse neuroblastoma Neuro-2a line	LePage et al. (2005)
2010–2012	Fibroblast conversion to multipotent neural cells by the expression of ASCL1, RN2A, and MYT1L and by SOX2	Vierbuchen et al. (2010) and Ring et al. (2012)
1998	Embryonic stem cells (ESCs)	Thomson (1998)
2001	Neuronal cell lineages differentiated using PSC technology	Reubinoff et al. (2001) and Zhang et al. (2001)
2003	Serum- and growth-factor-free methodologies to differentiate ESCs into neural precursors	Ying et al. (2003)
2005	Forebrain precursors generated from serum-free embryoid bodies on poly-D-lysine/laminin/fibronectin-coated dishes	Watanabe et al. (2005)
2007	Induced pluripotent stem cells (IPSCs)	Takahashi et al. (2006)
2009	Dual SMAD inhibition by noggin and SB431542	Chambers et al. (2009)
2011	Retinoic-acid-induced human pluripotent embryonic carcinoma stem cell neurons	Coyle (Coyle et al. 2011)
2012	Dual SMAD inhibition + Wnt signaling activation by GSK3	Kirkeby et al. (2012)
2013	Mature neurons generated by forced expression of Neurogenin-2 (or NeuroD1)	Zhang et al. (2013)
2008	Hypothalamus organoids by Nodal/Activin/TGF-β and BMP-mediated inhibition	Wataya et al. (2008)
2011	First self-patterned CNS organoid generates neuroepithelial cysts and optic cup organoids by nodal treatment and Matrigel basement membrane culture	Eiraku et al. (2011)
	Adenohypophysis organoids using hedgehog agonists	Suga et al. (2011)
2013	Extrinsic-patterning neocortical forebrain organoids by serum-free embryoid bodies treated with Wnt and TGF-β inhibitors on Matrigel	Kadoshima et al. (2013)
	Whole-brain/cerebral organoids displaying different brain regions generated from serum-free embryoid bodies cultured in low bFGF-2 concentration and ROCK inhibitors	Lancaster and Knoblich (2014)

(continued)

Table 1 (continued)

Year	Reports	Authors
2015	Retinoic acid stimulates neurogenesis by inhibiting Notch and Geminin and promoting proneural and neurogenesis genes	Janesick et al. (2015)
	Self-organized aggregates as an alternative 3D culture approach, encompassing neural spheroids	Dingle et al. (2015)
	Cerebellum organoids by Nodal/Activin/TGF-β inhibition and addition of GF2 and FGF19	Muguruma et al. (2015)
	Hippocampal-choroid plexus organoids by treatment with BMP and Wnt	Sakaguchi et al. (2015)
	Cerebral organoids display gene expression signatures of fetal developing neocortex	Camp et al. (2015)
2016	Modification of self-aggregating cultures into organ-like structures	Kelava and Lancaster (2016)
	Midbrain organoids by SMAD inhibition and Wnt activation	Jo et al. (2016)
2017	Long-term cerebral organoids display astrocytes and oligodendrocytes, mature glial cells, and gene expression profiles comparable to postnatal brains	Renner et al. (2017), Sloan et al. (2017), and Matsui et al. (2018)
2018	Neanderthal cerebral organoids generated by introducing the Neanderthal gene NOVA1 in human iPSCs	Cohen (2018)
	Organoids cultured in a rotatory system to observe microgravitational effects	Mattei et al. (2018)
	Generation of vascularized organoids to mimic BBB	Mansour et al. (2018), Nzou et al. (2018), and Pham et al. (2018)
	Organoids-on-chip	Tachibana and Miller (2018)

Abbreviations: *BBB* blood-brain barrier, *bFGF* basic fibroblast growth factor, *CNS* central nervous system, *ESCs* embryonic stem cells, *iPSCs* induced pluripotent cells, *NSCs* neuronal stem cells, *TGF-beta* transforming growth factor-beta

beneficial genes or combined transplantation with mesenchymal stem cells (MSCs) or their preconditioning with chemicals (Hosseini et al. 2018a, b) could provide a strategy for a successful therapeutic application by enhancing survival posttransplantation.

Even though much progress has been made to understand the basic biology and possible therapeutic applications of NSCs, still cell replacement technologies have certain limitations, such as the following:

- Scalability: handling and maintenance of a high volume of high-quality control cultures.

Table 2 Sources to isolate and culture neural stem cells

Origin	Concept	Advantages	Disadvantages
Embryo, usually left over from in vitro fertilization (IVF)	Derived from the inner cell mass of the blastocyst	Strong capacity to self-renew and is easily expandable, can be easily maintained in vitro or transformed into cell lines, can be used for the derivation of multiple cell types	Continued proliferative activity after transplantation, high risk of tumor formation, ethically controversial
Fetal, aborted between gestational weeks 6–20	Isolated from almost any part of the fetal human CNS	Committed toward neural phenotype, can be expanded and passaged numerous times without losing self-renewing and neurogenic capacity	Limited availability, ethically controversial
Adult brain	Isolated from neurogenic niche of adult brain or stimulated after injury to migrate and promote regeneration	Committed toward neural phenotype	Low viability in vitro after isolation, difficult to expand and maintain
Induced pluripotent stem cells	Reprogramming of somatic cells to pluripotent stem cells	Readily available sources, powerful capacity for proliferation, and differentiation in vitro; ethically uncontroversial	Continued proliferative activity after transplantation, high risk of tumor formation

- Reproducibility: structural complexity, and reproducing the architectures is still not possible.
- Vascularization: with limitations in vasculature formation in vitro, nutrition and growth factor supply for the cells in the inner core is limited.
- Blood-brain barrier: limiting the effective interaction of immune cells with the brain cell.

Influence of the Microenvironment and Niche on Embryonic NSCs – Intrinsic Regulation

In mammals, NSCs appear early in development and remain active in the CNS of the organism for a lifetime. During this process, NSCs retain their core stem cell features without undergoing differentiation supported by signaling molecules secreted from the niche milieu.

During early neural development, NSCs are termed neuroepithelial cells, and they are exposed to various signaling cues, including retinoic acid, sonic hedgehog, and fibroblast growth factors (FGFs). During neurogenesis, NSCs are transformed into

radial glial cells (RGCs). The complexity of their microenvironment increases further due to the emergence of various types of neuronal progenitors, differentiated cells, and extracellular signaling molecules. During adulthood, NSCs differentiate to achieve astroglial morphology and reside in specific microenvironments, which together constitute the neurogenic niche.

Both extrinsic and intrinsic mechanisms control the nature of NSCs/precursor cells. Studies have suggested that E10 cortical progenitors cultured clonally could generate neurons (neurogenesis) first and then glia (gliogenesis), the two important components of early neuroepithelium, a sequence that is exactly followed in vivo during CNS development (Qian et al. 2000). Another study has demonstrated that this intrinsic timer extends to the sequential development of different cortical neuron subtypes (Shen et al. 2006). Researchers have also demonstrated that progenitors maintain their intrinsic potential when grafted ectopically (Darsalia et al. 2007; Olsson et al. 1998).

Many transcription factors play a critical role in NSC/progenitor cell proliferation and/or differentiation, which include proneural genes, such as (a) basic-helix-loop-helix (bHLH) (Bertrand et al. 2002), (b) SRY-related HMG box (SOX) family (Episkopou 2005), (c) nuclear receptor estrogen receptor (Brännvall et al. 2002), (d) peroxisome proliferator-activated receptor γ (Wada et al. 2006), and (e) N-CoR, a nuclear receptor corepressor (Hermanson et al. 2002). Loss or gain of function of many of these transcription factors has been shown to alter progenitor specification, cell cycle, and cell fate, irrespective of the environment (Bertrand et al. 2002; Campbell 2003; Guillemot 2005). Epigenetic modifications/regulations, such as methylation, acetylation, and phosphorylation are implicated in controlling NSCs' intrinsic properties. Histone acetylation of neuronal gene promoters is modulated by histone acetyltransferases (HATs), and deacetylases (HDACs) are essential for the repression of these genes in undifferentiated neuronal progenitors (Ballas and Mandel 2005).

Signaling Molecules That Affect NSC Differentiation

The niche/microenvironment (extrinsic factors) regulates the behavior of neuronal progenitors through diffusible signals and/or molecules mediating through cell-to-cell and cell-to-ECM interactions. The signaling molecules, by their nature, circulate within the developing CNS tissue and can signal areas distant from their sources for a region-specific behavior. Among several molecules, bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGFβ) play critical roles in regulating the proliferation and differentiation of NSCs (Bertrand and Dahmane 2006; Campbell 2003).

Studies suggested that the overexpression of BMP 2 and 4 decreased neural stem cell proliferation and premature neuronal differentiation (Li et al. 1998), and the addition of noggin (BMP signaling inhibitor) inhibited the effect (Li and LoTurco 2000). The in vivo overexpression of BMP type I receptor promoted cell differentiation at the expense of cell proliferation (Li et al. 1998). Several fibroblast growth factors (FGFs) in general, and FGF8 and FGF3 in particular, are reported to be expressed in the anterior neural ridge, the midbrain-hindbrain barrier, and rhombomere 4 of the hindbrain early in development (Mason 2007). Studies have suggested the role of FGF

activity in controlling the patterning of the nervous system and aiding in the survival of cells in the forebrain and hindbrain region (Storm et al. 2003; Chi et al. 2003). Several diffusible molecules are essential in the regulation of neural precursor behavior. The following are examples:

a) Sonic hedgehog (Shh) signaling is mediated by its receptor patched (PTC1), a transmembrane protein (Dessaud et al. 2007), which in the absence of Shh constitutively represses the G-protein-coupled receptor smoothened (Smo). After binding with Shh, PTC1 relieves its inhibition on Smo and activates downstream signaling pathways, which result in the modulation of transcriptional activators Gli1–3 and the Gli repressor involved in regulating progenitor proliferation (Chiang et al. 1996; Muenke and Cohen 2000; Palma and Ruiz i Altaba 2004).
b) Retinoic acid (RA) is a diffusible molecule that is produced intracellularly by retinaldehyde dehydrogenases (RALDH1–3). RA is sequestered in the cytoplasm by cellular-RA-binding proteins (CRABP1–2) and acts in the nucleus after binding to RA receptors (RAR1–3) and retinoic X receptors (RXR1–3) (Maden 2002). RA is important in the very early neuronal microenvironment and participates in the regulation of the anterior-posterior axis (Maden 2002). In later stages, RA signaling is important for the dorsoventral patterning of the spinal cord (Pierani et al. 1999) and hindbrain (Marshall et al. 1992; Takahashi and Liu 2006).
c) The Wnt signaling pathway has also been shown to regulate cell behavior in the developing brain. Studies suggested that β-catenin signaling is necessary to maintain the ventricular zone (VZ) progenitor population and is downregulated when VZ progenitors are transitioning toward an intermediate progenitor (SVZ) fate. The sustained β-catenin activity resulted in the expansion of the VZ progenitor pool and inhibited the production of intermediate progenitors (Wrobel et al. 2007).
d) The Notch signaling pathway has a critical role in CNS development (Hitoshi et al. 2002; Yoon and Gaiano 2005). A strong expression of Notch-1 and Delta-1 and a weak expression of Notch-3 have been reported in the human VZ (Kostyszyn et al. 2004). Notch signaling also plays an essential role in cell fate determination since the activation of either Notch-1 or Notch-3 results in increased numbers of radial glia (Dang et al. 2006; Gaiano et al. 2000).
e) Cadherin-dependent adherens junctions (AJs) is another form of cell-to-cell interaction in the VZ, which might be mediated by cadherin signaling in the SVZ (Lathia et al. 2007a). The cell layer adjacent to the ventricle is characterized by strong cadherin expression in the apicolateral part of the cell membrane (Aaku-Saraste et al. 1996).

Extracellular Matrix (ECM) Role in Regulating Neural Stem Cell Characteristics

In addition to diffusible signaling molecules, ECM is another integral component of the VZ/SVZ microenvironment, which plays an essential role in regulating NSC/precursor behavior. The cell bodies and the short apical processes of NSCs/

progenitors are positioned in an area devoid of a classic basement membrane but still rich in matrix molecules, such as laminin chains (Campos et al. 2004; Hunter et al. 1992; Lathia et al. 2007a), the laminin receptor beta1 integrin (Campos et al. 2004) (Graus-Porta et al. 2001; Hall et al. 2006; Nagato et al. 2005), the glycoprotein tenascin-C (Garcion et al. 2004), and chondroitin sulfate proteoglycans (CSPGs) (Von Holst et al. 2006). In addition, many of the bipolar (neuroepithelial or radial glial) cells of the VZ extend a basal process that makes contact with the ECM-rich basement membrane of the pia. Alterations in this basal microenvironment have been correlated in humans with cortical malformations caused by migration defects (Bonneau et al. 2002) (Toda et al. 1994; Yoshida et al. 2001). The enzymatic degradation of CSPG glycosaminoglycans using chondroitinase ABC resulted in altered proliferation and neuronal differentiation, suggesting an essential role of ECM molecules in the regulation of NSC/precursor behavior (Sirko et al. 2007).

Cell-cell and cell-ECM signaling also regulate NSC and progenitor characteristics within adult niches. mRNA analysis revealed that members of the Notch signaling pathway (Notch and Jagged) are present in both the SEZ and SGZ (Stump et al. 2002). Ephrins and their receptors are present in adult neurogenic niches and regulate NSC proliferation (Conover et al. 2000; Holmberg et al. 2005). Intercellular interactions within the neuronal niches are thought to be mediated by cadherin-dependent adherens junctions or gap junctions formed by connexins. However, evidence for these interactions in other adult stem cell niches and the embryonic NSC microenvironment (Lathia et al. 2007b) and their functional relevance in adult NSC niches is to be established. The role of ECM molecules that are expressed in adult neurogenic niches is under further exploration. Tenascin-C, expressed in the SEZ, a glycoprotein that regulates growth factor activity during brain development (Garcion et al. 2004), was shown to be critical for the neurogenic process (de Chevigny et al. 2006; Kazanis et al. 2007). Kerever et al. (2007) reported that laminin-rich fractions could capture FGF2 and regulate growth factor concentrations and their activity across the SEZ (Bandtlow and Zimmermann 2000). The expression analysis of chondroitin sulfate, glycosaminoglycans (Sirko et al. 2007), and multiple chondroitin/dermatan sulfotransferases (Akita et al. 2008) also emphasized the role of ECM in NSC/progenitor characteristics.

Anomalies in Neurogenesis and Brain Pathologies

Developmental malformations, such as small (microcephaly) or large (hemimegalencephaly) brains, or focal abnormalities (focal cortical dysplasia) have been mainly attributed to the dysregulated production of neurons and/or glial cells during embryonic development, leading to conditions like mental retardation and epilepsy (Pang et al. 2008). The causative genes that have been correlated with the above-said pathologies are associated with intrinsic cell-cycle regulation rather than the extracellular microenvironment (Bond and Woods 2006). The published data strongly suggest that mutations in the four genes have been identified for autosomal recessive primary microcephaly, a

neurodevelopmental disorder that is characterized by the congenital occurrence of a small brain with normal cytoarchitecture, without progressive cognitive decline, and with seizures (Woods et al. 2005):

i) Microcephalin, a protein that plays a role in controlling cell-cycle timing
ii) Abnormal spindle-like, microcephaly associated (ASPM) gene, a protein that is important for the formation of the central mitotic spindle
iii) Cyclin-dependent kinase 5 regulatory associated protein 2 (CDK5RAP2), which interacts with gamma-tubulin ring complexes during spindle formation
iv) Centromere-associated protein J (CENPJ), which is important in microtubule nucleation and polymerization

Filamin A (FLNA) is reported to be associated with the ectopic occurrence of neuroglial clusters at the ventricular surface (periventricular heterotopia) and to cause newborn cells to migrate away from the ventricles (Lu et al. 2006). FLNA is highly expressed in the neuroepithelium adjacent to the ventricles and contributes to the structural integrity of the neuroepithelial layer. Genes that encode components of the microenvironment surrounding neuronal progenitors have been correlated with the loss of the gyri and sulci of the brain, which is termed lissencephaly (Pang et al. 2008). The absence of reelin (Bonneau et al. 2002), a signaling glycoprotein secreted by early-born neurons at the surface of the cortex, has been associated with this type of lissencephaly, although most human cases have been correlated with gene encoding for proteins regulating microtubule assembly, such as lissencephaly 1 (LIS1) (Reiner et al. 1993), Doublecortin (Dcx) (Gleeson et al. 1998; Pilz et al. 1998), and tubulin alpha 1A (TUBA1A) (Keays et al. 2007). Studies have also suggested that the depletion of presenilin 1 (Hartmann et al. 1998), alpha6 integrin, and integrin-linked kinase (Georges-Labouesse et al. 1998; Niewmierzycka et al. 2005) result in lissencephaly.

In another study, the pathology corresponding to cobblestone lissencephaly has been associated with the following four genes:

(i) Protein-O-mannosyltransferase 1 (POMT1)
(ii) Protein-O-mannosyltransferase 2 (POMT 2)
(iii) Protein-O-mannose 1,2-N-acetylglucosaminyl-transferase (POMGnT1) (Yoshida et al. 2001)
(iv) Fukutin (Toda et al. 1994)

All of these four genes are involved in the glycosylation of α-dystroglycan, a receptor for multiple ECM molecules. Mutations in the abovementioned genes result in alteration in the integrity of the basal lamina barrier at the pial surface, leading to the migration of cells (Guerrini and Marini 2006; Pang et al. 2008) (Table 3).

Neurogenesis during adulthood results in a limited generation of newly developed and functionally integrated cells and maintains tissue homeostasis in specific systems (Ming and Song 2011). However, there is mounting evidence suggesting that signals from neurogenic niches are responsive to local signals generated from

proximal tissue damage to remote areas because of changes in the external macro-environment of the tissue (Pino et al. 2017). Studies suggest that enhanced proliferation in the SEZ has also been reported in patients suffering from epileptic seizures (Grote and Hannan 2007) and multiple sclerosis (Nait-Oumesmar et al. 2007). They suggest that neurogenesis is significantly reduced in mood disorders, such as depression and stress (Grote and Hannan 2007). A study report demonstrated that ischemia results in the expansion of the SEZ, characterized by tenascin-C and with concomitant induction of hypoxic conditions and a transient decrease in vascular density (Thored et al. 2007). It is also reported that the altered expression of growth factors, morphogens, and ECM molecules occurs following a stroke episode (Liu et al. 2007). Moreover, the inflammatory signals in the SEZ are essential for regulating neurogenesis (Phillips et al. 2005). A recent study has reported that peripherally induced inflammation promoted the transient activation of primed NSCs, in which TNF-a has a significant role, acting via TNF receptors (TNFR) 1 and 2 (Belenguer et al. 2021). It has also been proposed to promote remyelination following demyelination, which could be a cause for repair in the mammalian CNS (Zawadzka and Franklin 2007) (Figure 1).

Advances in Neural Stem Cell Transplantation and Therapy (Fig. 1 and Table 3)

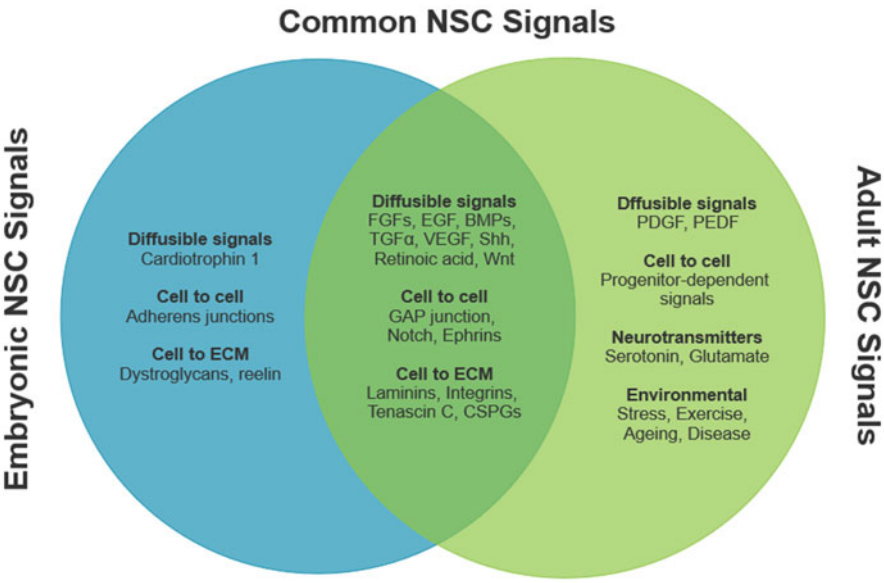

Fig. 1 Summary of the signaling pathways in the neural stem cell microenvironment (Kazanis et al. 2008)

Table 3 Update on the recent advancements in research on neural stem cells and disorders (da Silva Siqueira et al. 2021)

Target disease or disorder	Major findings
Alzheimer's disease	
Proliferation of neural progenitors	Due to the presence of β-amyloid plaques and the formation and proliferation potential of neurospheres in AD models, when compared to normal animals, in addition to lower cell index, stabilization, and expansion of neurospheres
Brain injury	
Transplanting neural progenitors	NSCs and NPCs from 10-week-old human forebrain showed great transplantable therapeutic application in case of traumatic brain injury as these cells survived in Sprague-Dawley rats, also demonstrating migration and proliferation in various regions of the brain, viz., hippocampus, corpus callosum, ipsilateral subependymal zone, and contralateral cortex
Degenerative diseases	
Tissue regeneration	Stem cells and progenitor cells from rodent olfactory bulbs can engraft in various neuronal organs, migrate, proliferate, and differentiate, becoming specific cell types for the tissue regeneration of damaged tissues
Demyelinating diseases	
Demyelinating spinal cord	NPCs from the adult human brain can generate functional cells and promote the remyelination of axons in a demyelinated rat spinal cord
Monitoring of glial progenitor cells	Magnetically labeled glial progenitor cells from a Lewis rat can be monitored for its migration and distribution into brain parenchyma using MRI
Cell proliferation and survival	Transplanted neurospheres are able to survive in the ventricles of mice and migrate and respond to inflammations caused. Due to this ability of neurospheres, they have clinical potential for transplants and therapies for demyelinating diseases
β4-tubulin	β4-tubulins are neural precursors that are sources for the last stage of myelination and for neural repair. In mice, upon transplantation, it shows activation, proliferation, and differentiation of β4-tubulin in the case of deficiency and dysfunction of oligodendrocytes
Differentiation of adipose-derived stem cells	Schwann cells differentiated using adipose-derived stem cells show similar morphology, phenotype, and functional capabilities and can be used for the treatment of neurological disease
Diabetes	
Cognition and memory	Hyperglycemic environment increases proliferation but decreases the survival of adult neural progenitors in SVZ and DG
Epilepsy	
TLE model and inflammation	Improvements in TLE rat models have been observed after transplantation of human NSPCs, including reduced frequency and/or duration of seizures

(continued)

Table 3 (continued)

Target disease or disorder	Major findings
Medial ganglionic eminence progenitors	Neurospheres cultured using cells obtained from MGE, upon differentiation, become inhibitory neurons. In vitro, they differentiate into inhibitory neurons and glial cells; in vivo, they reduce the frequency and duration of epileptic seizures
Oxidative damage	Neurospheres from Wistar rats showed a neuroprotective potential against seizures in animal models of epilepsy
Glioma	
Inhibition of glioma cell proliferation	Studies have suggested that NSPCs isolated from E14 mouse embryos secrete factors that inhibit the proliferation of glioma cells
Gliomagenesis and NSPCs and SVZ	The isolation of cells and neurospheres from SVZ isolated from rats showed altered phenotypic characteristics after 10–15 doublings, and they became immortalized. These can be considered a precursor for glioma and can be used as a model for generic and epigenetic investigations resulting in complete malignancy
Huntington's disease	
Human neural stem cell transplantation	Engraftment of neurospeheres derived from Human fetal tissue improved the differentiation of neurons and astrocytes and also protected the neuronal cell loss, in an HD model of rodent
Ischemia	
Neurogenesis	Neurospheres from the amniotic fluid source showed common features such as those of other NSC sources and have shown proliferation and differentiation capacity into astrocytes, oligodendrocytes, and dopaminergic neurons in vitro. They also showed therapeutic effects in ischemic rats, making a new source for human NSCs and for the translational studies of neurological disorders
Proliferation of postischemic NSCs	Adult Wistar rat induced with ischemia exhibits a higher neurogenic rat than do nonischemic rats, suggesting that the stroke increases NPC proliferation
Neurodegenerative disorders	
Transplanting neural progenitors	HNPCs can be maintained and expanded in culture and can be differentiated in order to transplant them. These cells show high survival, migration, and transplantation rates in rats with striated lesions
Parkinson's disease	
Human neural progenitor cell transplantation	HNPCs have shown promising results in case of treatment of Parkinson's disease when these cells were transplanted in adult Lewis rats with a partial lesion of Parkinson's disease
GABA receptors	$GABA_B$ receptor activation decreases and $GABA_A$ receptor activation increases the number of dopaminergic neurons generated from neurospheres. The use of $GABA_A$ receptor antagonists helps obtain a greater number of neurons for potential use in cell therapy for PD

(continued)

Table 3 (continued)

Target disease or disorder	Major findings
Dopaminergic neurons	Use of dopaminergic neurons from any stem cell source has great potential for clinical application for PD as they show high survival and migration rates and significant functional benefits, resulting in synaptic improvement Multipotent NSCs have, upon transplantation, also showed the ability to migrate and differentiate in TH-positive neural cells without any use of immunosuppressant.
Pediatric brain tumors	
Multipotent tumor cells	Progenitor cells derived from pediatric brain tumors show similar characteristics of the NSCs isolated from CNS, with migration and proliferative ability when transplanted into the brain of neonatal rats. These results suggest that PBTs have self-renewable cells, with altered characteristics giving rise to tumorigenesis
Spinal cord injury	
Transplanting neural progenitors	Spinal cord NSPCs have shown the ability to survive and differentiate into astrocytes, oligodendrocytes, and a small number of neurons, upon transplantation to Sprague-Dawley rats with acute spinal cord injury model
Axonal regeneration	Biodegradable polymers seeded together with NSCs and Schwann cells facilitate regeneration across the transected spinal cord
Striatal injuries	
Intracerebral graft	NSPCs represent a long-term expandable source of cells for potential use in intracerebral grafts
Stroke	
Atorvastatin	Therapy with atorvastatin in Wistar rats poststroke increases the proliferation of NPCs
P53 PFT-α inhibitor	Treatment with p53 PFT-α inhibitor modifies brain injury and induces neurogenesis, improving the proliferation and survival of NPCs in SVZ in animals after stroke
Poststroke recovery, inflammation, and vascular repair	HCNS-SCns, upon transplantation, suppress inflammation after stroke, increase vessel formation, and improve the integrity of the blood-brain barrier, positively interfering with vascular repair after inflammation
Erythropoietin	Treatment of Wistar rats poststroke with erythropoietin significantly improves functional recovery, angiogenesis, and neurogenesis in animals, helping increase neurological functions

Abbreviations: *CNS* central nervous system, *hNPCs* human neural progenitor cells, *NPT* neuronal progenitor cells, *NSCs* neuronal stem cells, *PD* Parkinson's disease, *SVZ* subventricular zone, *TLE* temporal lobe epilepsy

Conclusions

Although it is easy to identify altered neurogenesis and/or migration as the leading cause of developmental brain malformations (Guarnieri et al. 2018), it is very complicated and technically challenging to directly address whether defects in adult neurogenesis can also cause pathologies. This is because samples from patients are mostly obtained only after a disease has been diagnosed, thus making it difficult to distinguish the cause from effect. Experiments where neurogenesis is genetically disturbed, specifically in the postnatal brain, are lacking; only exogenously induced perturbations (i.e., irradiation, growth factor injections) have been studied, and these studies, too, are only limited to their short-term effects. Despite significant progress, technologies to position NSCs for more comprehensive clinical applications are still not mature and established for routine use as a therapeutic option. The keystone in cell-based regenerative medicine is to identify the specific factors that could help in the promotion of cell adhesion, growth, and differentiation into distinct and desired lineages.

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Glial Cells in Neuroinflammation in Various Disease States

28

Derek Barthels and Hiranmoy Das

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Abstract

With current advancements in central nervous system (CNS) research, it is becoming increasingly obvious that glial cells play important roles in both physiology and pathology in the CNS. This chapter focuses on microglia and astrocytes, as each supports inflammatory functions and neuronal health in the CNS. Given the lagging behind of this field of study, research is currently being conducted to elucidate the roles of these cells in CNS pathologies, such as stroke and its aftermath, Parkinson's disease, and Alzheimer's disease. As current therapy does not cure these diseases, we evaluate a potential new therapy using human adult stem cells that could help treat such conditions, as they are well documented to have potent anti-inflammatory activity and are able to produce

D. Barthels · H. Das (✉)
Department of Pharmaceutical Sciences, Jerry H. Hodge School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, TX, USA
e-mail: hiranmoy.das@ttuhsc.edu

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factors such as glial- and brain-derived neurotrophic factors to support neuronal health. It is entirely possible that this therapeutic strategy could serve as a multifaceted approach, as the support through growth factors and the increased control of neuroinflammation could support astrocyte health, contributing to the maintenance of literally millions of neural synapses. Herein, we will discuss in detail how microglia and astrocytes contribute to different CNS pathologies and examine the potential efficacy of a human adult stem cell–based therapeutic approach that targets these cells.

Keywords

Astrocytes · CNS disorders · Microglia · Neuroinflammation · Stem cells

Abbreviations

A20	Ubiquitin-modifying protein A20
AD	Alzheimer's disease
APOE	Apolipoprotein E
AQP4	Aquaporin-4
Aβ	Beta amyloid
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CAS9	CRISPR-associated protein 9
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CRYAB	αβ-crystallin
CSPG	Chondroitin-sulfate proteoglycan
DPSC	Dental pulp stem cell
ECM	Extracellular matrix
GABA	Gamma aminobutyric acid
Gal-9	Galectin-9
GDNF	Glial-derived neurotrophic factor
HD	Huntington's disease
IL	Interleukin
INF	Interferon
M1	Pro-inflammatory
M2	Anti-inflammatory
MHC	Major histocompatibility complex
mHTT	Mutant huntingtin protein
miRNA	microRNA
MMP	Matrix metalloproteinases
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MTPT	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF-κB	Nuclear factor-kappa B
NMDA	N-methyl-D-aspartate

NMO	Neuromyelitis optica
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSAID	Nonsteroidal anti-inflammatory drug
NSC	Neural stem cell
PD	Parkinson's disease
siRNA	Small interfering RNA
SSRI	Selective serotonin reuptake inhibitors
TALENS	Transcription activator-like effector nucleases
TBI	Traumatic brain injury
TGF	Transforming growth factor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TREM2	Triggering receptor expressed on myeloid cells 2
VEGF	Vascular endothelial growth factor
ZFN	Zinc-finger nucleases

Introduction

Both neurons and glial cells were first identified in the mid-nineteenth century, but neurons initially received major attention in research, and investigations of glial cells were not carried out in earnest until the 1950s. One of the main obstacles in earlier glial cell research was the lack of reliable methods to analyze glial function and signaling, despite the early observation of morphological changes of star-shaped glial cells (eventually named astrocytes) in central nervous system (CNS) disorders. Additionally, microglial cells, another subset of glial cells, were not identified until 1919 (Fan and Agid 2018). Due to the lack of methods to analyze astrocytes and microglia, in vitro models were developed that investigated neuronal function isolated from other cells normally present in the CNS, and these glial cells were considered nothing more than "glue cells" of the CNS (Jakel and Dimou 2017). While these models are valuable and essential for understanding neuronal function, the absence of other cells native to the CNS can drastically alter how the cells function. However, studies conducted over the last century have shown astrocytes and microglia, and their elucidated functions, to be instrumental in the overall function and structure of the CNS, and in the development of several CNS pathologies (Falk and Gotz 2017).

Microglial Cells and Their Physiological Functions

Microglial cells make up 5–12% of the cells in the CNS and serve as the innate immune cells for this immune-privileged system (Hickman et al. 2018). Much of the earliest research on microglia was carried out by the Spanish neuroscientist Pío del Río Hortega in the early 1900s, but research on these cells did not reach full steam until the end of the century (Somjen 1988). Since they are the resident macrophages

of the CNS, microglial cells are tasked with the identification and destruction of foreign pathogens from the CNS. When functioning properly, microglia also acts to mitigate the damage caused by CNS insults by clearing dead and dying tissue in the aftermath of an injury. This process primes the site for healing mechanisms that occur later to repair the damage done (Wolf et al. 2017). To better accomplish these facets of their purpose, microglia are constantly "scanning" their environment for signals indicating either infection by a pathogen or for markers indicating the need for intrinsic elements of the CNS to be broken down. This process is partially responsible for facilitating neural plasticity (Kettenmann et al. 2011). Even though microglia are carrying out CNS-preserving activities in this state, this scanning state is known as the "resting" state. These cells are characterized by low expression of specific surface markers, including CD45, major histocompatibility complex (MHC)-II, and CD11b. Upon activation, microglial cell bodies undergo hypertrophy, and the cells' surface markers are upregulated, as well as intracellular markers such as Iba1 (Ma et al. 2017). In this activated state, microglia also have an increased capability to migrate within the CNS (Tay et al. 2017). This migratory ability is essential so that microglia can localize at the necessary site and carry out their roles in the aftermath of CNS insults. Despite the high amount of plasticity that microglia contain upon their activation, they have an innate memory of their homeostatic state and are able to revert to their inactive phenotype and continue to scan their microenvironment for the next job that needs to be completed (Matejuk and Ransohoff 2020).

In addition to their roles as macrophages, microglia play essential roles in both CNS development and are the primary instruments used to carry out neural remodeling. They can do this by pruning immature neurons and breaking down specific neural networks based on activity (Fields et al. 2014; Garaschuk and Verkhratsky 2019). This function is active both in early CNS development, and in cognitive and memory development in adults. They also contribute to the maintenance of homeostasis of CNS tissue. Though initially grouped with other glia as "glue cells," whose only purpose was believed to act only as scaffolds for networks of neurons, microglia are now realized more broadly as one of the primary drivers of CNS development (Allen and Lyons 2018). This can be attributed again to their sensitivity to their signaling microenvironment. Based on signals received, microglia can refine neural circuitry, provide trophic support to CNS cells, clear cellular debris, or destroy foreign pathogens. Current studies confirm microglia as major players in the complex processes of neurological development, and dysfunction of these cells is heavily implicated in developing several CNS pathologies, as discussed in depth later in this chapter (Cowan and Petri Jr. 2018).

Physiological Roles of Astrocytes

In healthy tissue, astrocytes serve as physical barriers to the infiltration of foreign cells and contaminants from blood vessels or the meninges. This barrier also contains the spread of neurotoxic inflammation that can spread from either damaged

CNS tissue or peripheral infections. Additionally, astrocytes serve to chemically regulate the permeability of the blood-brain barrier (BBB) (Sofroniew 2015). One factor produced abundantly by astrocytes is apolipoprotein E (APOE), a factor instrumental in maintaining the integrity of the BBB (Montagne et al. 2020; Rawat et al. 2019). Polymorphisms in the *APOE4* gene are correlated with poorer prognoses of conditions such as Alzheimer's disease (AD), stroke, and traumatic brain injuries (TBI) (Main et al. 2018; Safieh et al. 2019). Regulation of BBB permeability plays a central role in processes such as the recruitment of leukocytes. The process of extravasation for leukocytes in the CNS is similar to that in other parts of the body, but they must penetrate the BBB, which in and of itself is a complicated feat to accomplish (Manda-Handzlik and Demkow 2019). Retraction of astrocyte feet and the active recruitment of the leukocyte by secretion of signaling molecules from astrocytes and other cells facilitate the penetration of leukocytes through the BBB into CNS tissue (Sweeney et al. 2018). These functions also give astrocytes the ability to influence lymphocyte recruitment in perivascular spaces in the CNS. In this way, astrocytes' effects on the CNS can also be viewed as pro- or anti-inflammatory based on whether they promote or deny leukocyte infiltration through this mechanism. However, astrocytes can also directly influence inflammatory signaling by the secretion of inflammatory factors such as nitric oxide and prostaglandins. The induction of this state could lead to cytotoxicity and death of surrounding cells (Burda et al. 2016).

As important as the role of astrocytes is in the maintenance of the BBB, they also are instrumental in its repair after injury. These functions come in conjunction with the secretion of factors that directly promote repair, such as sonic hedgehog, or by suppressing factors, such as matrix-metalloproteinase-9 (MMP-9), that increase BBB permeability (Alvarez et al. 2011; Liebner et al. 2018). Two of the most influential pathways that mediate this effect include the gp130-JAK2-STAT3 pathway, as well as the dopamine D2 receptor pathway. Activation of these pathways can lead to the induction of factors such as retinoic acid and transforming growth factor (TGF)-β. Astrocytes even have the capability to provide anti-inflammatory support in pro-inflammatory environments, as the anti-inflammatory factors αβ-crystallin (CRYAB), galectin-9 (Gal-9), and ubiquitin-modifying protein A20 (A20) can all be induced by tumor necrosis factor (TNF)-α, interferon (INF)γ, or interleukin (IL)-1β (Sofroniew 2015).

In addition to the function of this physical barrier, hypotheses implicate astrocytes in the processing and transduction of neural signals. The introduction of these hypotheses initially created a great deal of controversy in the field of astrocyte research (Bazargani and Attwell 2016). However, with recent discoveries, the issues in question have mostly been resolved. Analysis of ion channels, including gamma-aminobutyric acid (GABA) and Ca^{2+} channels, and their roles have gone a long way in explaining the role that astrocytes can play in neurotransmission (Allen and Eroglu 2017). These more recent developments have inspired new approaches to analyze astrocyte function in the context of neurotransmission, primarily through the analysis of the role of intracellular Ca^{2+} and how it affects astrocyte function and CNS interactions.

Reactive Gliosis in Astrocytes

Many of the functional changes seen in astrocytes happen during a process called reactive gliosis. During this process, astrocytes undergo changes in gene and protein expressions, as well as changes in their morphology. These are the changes that allow for the infiltration of leukocytes after CNS trauma, as well as the formation of the glial scar that limits the damage around the lesion site. This process acts on a gradient of severity and is not a binary change in the cells (Sofroniew 2009). Astrocytes immediately adjacent to the lesion site will undergo the most severe form of gliosis, leading to permanent glial scarring. As the distance from the lesion site increases, the severity of the gliosis seen in astrocytes decreases. With decreasing severity, it becomes more likely that astrocytes will revert to their original phenotypes and carry out their processes normally (Sofroniew 2009). Reactive gliosis, when properly regulated, promotes the recovery of healthy tissue and is an essential step in the overall healing process. The reversible nature of reactive gliosis opens the possibilities for astrocyte-targeted therapies geared toward the regulation of gliosis, as preserving positive functions that can be lost with more severe gliosis states can help promote healthy function in the CNS (Colangelo et al. 2014).

Though glial cells were originally viewed as part of the supporting cast in the operation and development of the CNS, it is becoming more evident that these cells play a lead role and have a significant influence on the interactions of different parts of the system. It is well established that astrocytes and other glial cells play central roles in learning and cognitive processes (Augusto-Oliveira et al. 2019; Freeman 2010). To put the broad reach of these cells into perspective, one astrocyte has the astounding potential to interact with millions of neural synapses, and play central roles in the development and construction of new synapses (Fields et al. 2014). This ability of glial cells to contribute to neural plasticity and development can no longer be ignored. A shift in the way we frame CNS interactions could prove extremely beneficial in combatting various neurodegenerative diseases and general CNS trauma, for which effective treatments have proven to be elusive. A perspective of the CNS that regard glial cells as equal agents in CNS function could provide new avenues to manipulate potential therapeutic targets, such as astrocytes and microglia themselves. This chapter will discuss the latest research involving astrocytes and microglia as well as their roles in various disease states, giving a broader perspective on current research on novel therapeutic approaches for CNS disorders.

Stroke, CNS Trauma, and Their Impact on Glial Cells

It is well established that one of the primary roles of astrocytes is to respond to CNS trauma and to help regulate the immune response around the lesion site. The end feet of astrocytes that make up portions of the BBB will retract, drastically increasing the permeability of the BBB. This process leads to the recruiting and infiltration of leukocytes and lymphocytes into the CNS. In an attempt to control the onset of inflammation at the lesion site, astrocytes will form a glial scar around the site to

prevent the spread of potentially cytotoxic factors that result from the immune cells' activities (Adams and Gallo 2018). Inhibition of glial scarring has been associated with additional complications poststroke, such as increases in lesion size, increases in neuronal death and loss of functions, as well as the spread of uncontrolled inflammation (Wang et al. 2018). These consequences lead to the inhibition of axonal growth and regeneration and have the potential to negatively affect recovery after stroke. While astrocytes play significant roles in the clearance and containment of necrotic tissue, and inflammatory factors, there is evidence that they can be polarized toward harmful inflammatory states themselves, leading to increased cytotoxicity in the regions surrounding the ischemic core. Astrocytes in this state could still influence functional neurons and could cause additional damage after the initial injury (Neal and Richardson 2018). Increased secretions of chondroitin-sulfate proteoglycans (CSPGs) are also recognized as a characteristic of reactive gliosis. These factors act as roadblocks for axonal outgrowth and high levels of CSPGs can negatively affect recovery capability. Reactive astrocytes have been shown to produce different proteoglycans based on the type of linking group attached to the molecule (Silver and Miller 2004). Astrocytes produce four different classes of proteoglycans, with CSPGs being the subject of primary focus, as they are upregulated in glial scars within 24 h after insult to the CNS and persist for months after the injury (Silver and Miller 2004). The presence of proteoglycans can be detrimental to recovery after CNS insult. They have been identified as potent inhibitors of axon outgrowth in vitro. They are believed to stunt axonal outgrowth in the embryonic development of the CNS in zebra fish and mice (Silver and Miller 2004).

Microglial cells are also recruited to aid in the recovery process in the aftermath of strokes and other types of CNS trauma. Their inflammatory response is essential in laying the groundwork for subsequent infiltration of peripheral immune cells (Eldahshan et al. 2019). This activation can occur as early as 24 h after an ischemic event, and activated microglia can remain in this state for up to 150 days. The activated microglial cells are active in the infarct and peri-infarct regions after injury and behave as a double-edged sword. Microglial secretions can contribute to the damage and death of neurons. The heavily pro-inflammatory response seen in microglia also can affect the function of astrocytes and can lead to more severe reactive gliosis. This pro-inflammatory environment is caused by cytokines such as IL-1β and TNF-α from microglia and infiltrating leukocytes. While this aspect of the microglial response is necessary, it can run rampant in the CNS and reach a chronic, unresolved inflammatory state (Rothhammer et al. 2018). Chronic inflammation is associated with a bevy of secondary complications and is a high-priority problem that must be resolved.

One strategy to accomplish this feat is by manipulating microglial polarization. Like peripheral macrophages, microglia can be polarized toward M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes, and, like the reactive gliosis process in astrocytes, it is necessary to have a balance of M1 and M2 microglia when dealing with CNS insults. M1 microglia are most commonly seen in the aftermath of an injury, but the polarization of microglia to M2 phenotypes can

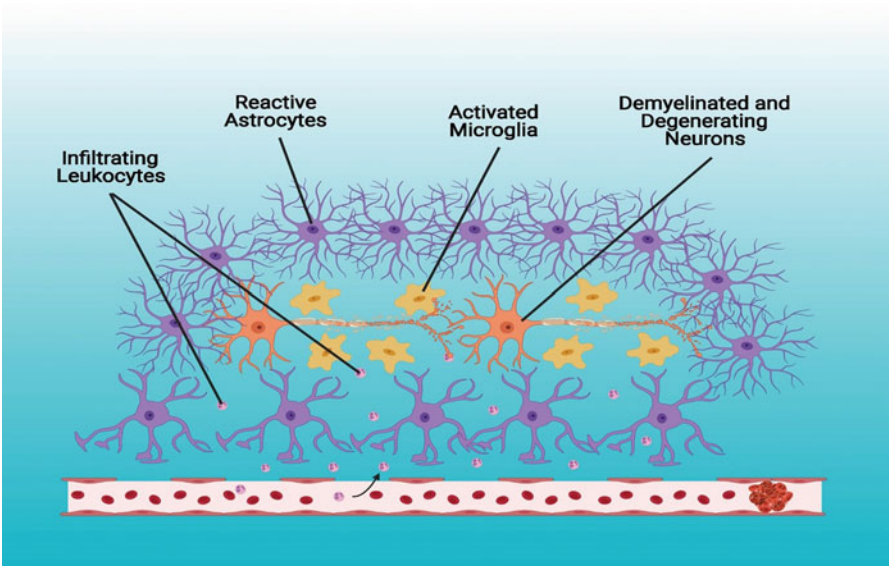

Fig. 1 The CNS in the aftermath of a stroke. After a stroke, the neurons at the epicenter of the stroke start to degenerate, while additional astrocytes move the lesion site and undergo gliosis to form a scar barrier. Excessive glutamate is in the extracellular space, leading to additional excitotoxicity in neurons. Microglia get activated to remove damaged tissue and clear space for healing processes, while infiltrating immune cells support the process after moving through the blood-brain barrier, which opens in part due to higher expression of matrix metalloproteinases

help keep inflammation from becoming chronic. M2 microglia can secrete potent anti-inflammatory factors such as IL-10, arginase, and YM-1. In addition to their potential for M2 polarization, microglia can also contribute to neuronal regeneration via the secretion of key growth factors, such as brain-derived neurotrophic factor (BDNF) (Lan et al. 2017). The ability to control the expression and secretion of these factors, along with regulating astrocyte function, may be a key to unlocking increased regenerative capacity in the aftermath of neuronal trauma **(**Fig. 1**).**

Roles of Glial Cells in CNS Autoimmune Conditions

Several experiments have also shown astrocytes to be central mediators and inhibitors of autoimmune inflammatory reactions in the CNS. One mechanism involves the targeting of aquaporin-4 (AQP4) on astrocytes. The binding of autoantibodies to these sites marks the astrocytes for lysis through the complement immune pathway. This condition, called neuromyelitis optica (NMO), can lead to the demyelination of the CNS in afflicted regions and can cause blindness and paralysis (Huda et al. 2019). The discovery of this specific origin of NMO led to clinical observations of higher severity in NMO patients who tested positive for anti-AQP4 antibodies

compared to those who only tested positive for anti-myelin antibodies (Sofroniew 2015).

Additionally, astrocytes may play a role in developing multiple sclerosis (MS), though the mechanisms regarding MS are currently not well understood. Early hypotheses posit that specific polymorphisms that occur in astrocytes could influence an individual's susceptibility to MS, based on their ability to keep the BBB intact and contain the spread of the auto-inflammatory response (Sofroniew 2015). Despite the ambiguity in exact mechanisms, the astrocyte end feet that form part of the BBB are one of the first casualties of the disease. This early damage likely has downstream effects on astrocytes' abilities to limit inflammation in these regions (Ponath et al. 2018).

A unique feature of both of these autoimmune conditions is their increased prevalence in women compared to men. It is estimated that for every man that has NMO, seven women live with the condition, and two women have MS for every man that has it (Sofroniew 2015). Given recent revelations regarding the difference in basic neurological function between men and women, such as how pain signals are propagated through the nervous system, it is not improbable that there are other differences that can contribute to the increased prevalence of these cases in women (Sorge et al. 2015). Investigations into whether astrocyte function differs between men and women may offer further insights into why women are disproportionately affected by these conditions (Sofroniew 2015).

As the primary immune cells in the CNS, microglia are also heavily implicated in developing autoimmune diseases in the CNS. It has been demonstrated that although the initiation of NMO is associated with the binding of antibodies to AQP4, microglia are likely the ones to mediate the inflammatory attack, as the disease did not progress with the ablation of microglia in a murine model (Chen et al. 2020). It is worth emphasizing again that these conditions often can cause a positive feedback loop, with inflammatory signals amplified by the cross talk between different cell types in the CNS, such as that between astrocytes and microglia during the progression of NMO. In the cited study, physical interactions between microglia and astrocytes, as well as the implementation of the complement immune reaction pathway marks microglia as potentially pivotal therapeutic targets in NMO. The initiation of the complement pathway has been linked to neurotoxicity in the vicinity of astrocytes when serums containing either AQP4 bound IgG or various complement factors, including C1q and C6. When these factors were absent and complement inhibitors were added, neurons near astrocytes were preserved (Duan et al. 2018). Controlling inflammatory signaling, including the complement pathway, could prevent the development of neuronal lesions that often lead to the blindness and paralysis seen in NMO **(**Fig. 2**).**

MS is associated with heavy microglial activation and has been one of the conditions in which microglial autoimmune functions are most commonly investigated. Though other CNS trauma conditions also feature microglial activation, they also involve higher levels of peripheral macrophage infiltration and activation, which make the microglial activity harder to detect. MS features different lesion patterns and multiple tissue-damaging mechanisms, but all of these lesion sites

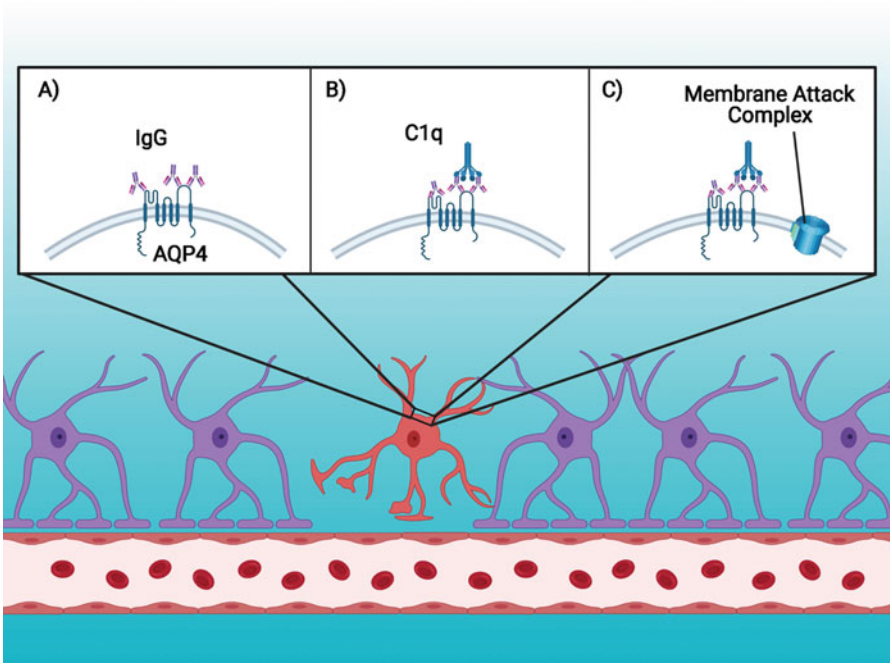

Fig. 2 Autoimmune mechanism involved in NMO pathology. One of the main phenomena seen in the development of NMO includes the autoimmune attack of AQP4 receptors on astrocytes. **A.** This process begins with the tagging of AQP4 receptors with IgG. **B.** This leads to the recruitment of the C1q factor in the complement pathway of the immune system. **C.** After the recruitment of subsequent subunits, a membrane attack complex penetrates the plasma membrane of the cell, leading to the lysing of the astrocyte

feature the demyelination coupled with neuroinflammation that is characteristic of the disease (Voet et al. 2019). The disruption in homeostatic microglial characterizations leads to highly pro-inflammatory regions where MS plaques continue to expand. Interestingly, M2-polarized microglial cells are present in the lesion cores in MS, and attempts at remyelination of these regions are common. These M2 cells are almost completely absent in the regions where plaques are progressing, and attempts at remyelinating these regions do not occur (Zrzavy et al. 2017). Another study asserts that the M2 polarization of microglia and macrophages is necessary to facilitate remyelination of the CNS in disease states like MS. It has been demonstrated that M1 and M2 phenotypes of microglia and macrophages can affect the differentiation of oligodendrocytes, thereby affecting how effectively they can facilitate remyelination (Miron et al. 2013). Restoring the balance between M1 and M2 phenotypes in microglia and macrophages could be the key to getting progressing and remissive MS under control.

Both NMO and MS are characterized by uncontrolled, chronic inflammation. The positive feedback loop of inflammation, leading to cell damage, which leads to more inflammation, needs to be interrupted through efficacious treatment strategies.

A strategy that is becoming more popular is manipulating the polarization of microglia to favor their M2 phenotype, thus restoring the balance between pro- and anti-inflammatory mechanisms. Theoretically, this approach will minimize the amounts of chronic, unresolved inflammation, which leads to the myelination issues seen in these autoimmune diseases.

Roles of Glial Cells in Neurodegenerative Disorders

Astrogliosis is a prominent factor in many neurodegenerative conditions, including AD and Huntington's disease (HD), and very well may be essential for the progression of many other neurodegenerative disorders. Experiments have shown that the inhibition of astrogliosis can potentiate the accumulation of β-amyloid (Aβ) plaques and increase inflammatory signaling (Frost and Li 2017). Reactive gliosis occurs in AD, and intriguingly, all the reactive astrocytes seem to be collected around Aβ deposits. One possible reason for this phenomenon may be that astrocytes possess receptors for advanced glycation end products, lipoprotein receptor-related proteins, membrane-associated proteoglycans, and scavenger receptor-like receptors. These receptors can interact with Aβ, leading to further recruitment of astrocytes and immune cells to the lesion site (Fakhoury 2018). These interactions can activate the nuclear factor-kappa B (NF-κB) pathway and initiate complement signaling, leading to the production of TNF-α and IL-1β and the perpetuation of the pro-inflammatory signaling environment seen in AD (Fakhoury 2018). Additionally, polymorphisms in the *APOE4* allele are linked to increased risk of AD and increased inflammation in the CNS (Sofroniew 2015).

The exact role of astrocytes in AD is still being elucidated. Some in vitro studies have suggested that reactive astrocytes are recruited to break down Aβ deposits in an effort to stave off the downstream effects of AD. In rodent models similar to AD, this process was carried out by neprilysin and insulin-degrading enzymes, and it is hypothesized that MMP-9 may also play a role. Conversely, other studies have shown that astrocytes could suffer from dysfunction after engulfing Aβ deposits that are not fully broken down. This result can lead to harmful apoptosis signaling in neurons. Additionally, astrocytes can deposit Aβ in pro-inflammatory environments, further complicating issues seen in AD (Fakhoury 2018). More studies must be conducted to fully elucidate how astrocytes behave in AD, and as these questions are answered, more targeted therapies could become available for AD. As seen in CNS trauma and autoimmune diseases, imbalance in astrocyte functions seems to be one issue contributing to the progression of the disease state, and restoration of proper astrocyte functions as a therapeutic approach may provide more efficacious prevention and recovery from AD.

Astrocytes are also implicated in the progression of HD. HD consists of the amalgamation of mutant huntingtin protein (mHTT) in astrocytes and neurons. Consequently, there is decreased expression of K^+ channels on these cells, leading to an increase in extracellular K^+ and increased excitability of neurons, potentially worsening HD (Khakh et al. 2017). Additionally, glutamate transporters such as

EAAT2 are not as abundant on astrocytes in HD, contributing to the excitotoxicity of neurons in the disease. This effect seems to be downstream of the expression and accumulation of mHTT in astrocytes, leading to the introduction of oxidative stress (Palpagama et al. 2019). These issues in astrocytes also trigger the NF-κB pathway in the astrocytes, contributing to the inflammatory environments that exist in HD. The effects observed in the CNS in HD are seen even before astrogliosis can be observed in experimental models, establishing reactive gliosis and astrocyte dysfunction as two discrete phenomena that are independent of one another (Khakh and Sofroniew 2014). However, the reactive gliosis that can be seen in Huntington's disease could contribute not only to neuronal death but also to pericyte death around cerebral blood vessels. This could potentiate the development of the disease. The exact mechanisms of how reactive gliosis affects the progression of HD have yet to be fully elucidated, as the number of studies done directly linking the two subjects beyond correlation with severity is scarce (Palpagama et al. 2019). A deeper analysis of these conditions and the roles that astrocytes play puts to rest the misconception that astrogliosis is an exclusively harmful occurrence either in the aftermath of CNS trauma or in the case of neurodegeneration **(**Fig. 3**).**

Inflammatory conditions in CNS disorders are prevalent, and AD is no different in this regard. One function of microglia is to engulf damaged cells and tissue, and

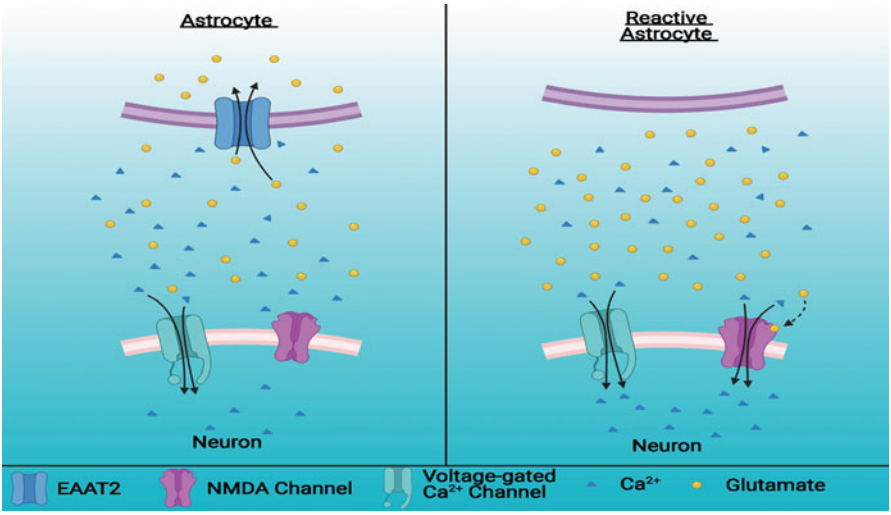

Fig. 3 Glutamate excitotoxicity in Huntington's disease. Healthy astrocytes typically have EAAT2 channels on their membranes, which are responsible for the uptake of excess glutamate in the extracellular spaces around neurons. This prevents the opening of excessive numbers of NMDA channels by glutamate binding, which allow the intake of additional Ca^{2+} ions by neurons. The opening of too many NMDA channels leads to the excessive influx of Ca^{2+} and hyper-excitability or even excitotoxicity in neurons. When astrocytes undergo reactive gliosis as seen in Huntington's disease, they express fewer EAAT2 channels on their membrane. This leads to excessive amounts of glutamate, which in turn opens more NMDA channels, causing excess excitability and excitotoxicity in neurons

they do so often via the complement pathway. Dysfunction of this pathway with microglia is heavily associated with AD. Mutations in these receptor genes, among other genes, often lead to hyperactivity of microglia, which engulf functional neurons. The hyperactivity of microglia is especially prevalent around Aβ aggregates (Hansen et al. 2018). These developments are heavily associated with genetic variation in the triggering receptor expressed on myeloid cells 2 (*TREM2)*, a gene almost exclusively expressed in microglia (Condello et al. 2018). Evidence suggests that when functioning properly, the *TREM2* gene enacts neuroprotective effects in the CNS, and it is theorized that the function of *TREM2* is affected by changes in inflammatory homeostasis as an individual gets older (Raha et al. 2017). *TREM2* is essential to combat toxicity at the beginning stages of AD, and the deficiency of this gene limits the ability for interactions between microglia and Aβ plaques. If indeed *TREM2* dysfunction is related to the development of a more pro-inflammatory environment, limiting the chronic inflammation could be preventative for loss of *TREM2* function, but further investigation is required to solidify the link between these factors.

Current Therapies for CNS Disorders

Many of these CNS pathologies have proven challenging to treat and innovative solutions to better help patients with these conditions are constantly being proposed.

Some effective therapies have been developed, such as applying recombinant tissue plasminogen activator (tPA) for the clearance of ischemic strokes. However, many of these treatments come with important caveats, such as the fact that the therapeutic time window to administer tPA to a patient is minimal (Faiz et al. 2018). Every medication comes with some drawbacks, but as many of these treatment strategies are in their infancy, some of them possess drawbacks that reduce the number of people eligible to receive them. In contrast, others have yet to produce efficacious results in clinical settings at all. With the difficulties seen in developing these treatments, refinement of existing strategies and development of novel approaches will be required to make notable progress in the clinical treatment of these conditions. Innovations are being developed to better assess and improve patient eligibility for various therapies, and new treatments are constantly being explored in research that put cell-based therapies at the forefront (Liaw and Liebeskind 2020).

Treatments for Stroke and CNS Trauma

Facilitating functional recovery in the aftermath of traumatic CNS injuries has proven to be highly elusive. One of the focuses of ischemic stroke treatments has been to restore circulation as soon as possible after the stroke occurs. As mentioned above, the application of tPA is effective within a window of 4.5 h after the stroke (Emberson et al. 2014). For patients that are ineligible for tPA, mechanical

thrombectomy is required if the clot does not clear by itself. While these treatments are helpful in breaking up the clot causing the stroke, the damage is often already done by the time circulation is restored (Lambrinos et al. 2016). This is where the innovation of treatments is needed to help restore functions lost in the immediate fallout of the stroke. Therapies that are used to help treat the aftermath of stroke will likely help those with other forms of CNS trauma, as similar pathologies develop with different presentations. As the common culprits in stroke and TBI are pro-inflammatory factors and deregulated permeability of the BBB, agents proposed to limit these mechanisms while boosting neuroprotective effects should prove to be effective candidates (Abdullahi et al. 2018; Anrather and Iadecola 2016). It is believed that more tightly regulating these processes, especially in the peri-infarct region of the lesion, will alleviate these symptoms. Adding to the rationale for these treatments is the abundance of factors that cause the breakdown of the extracellular matrix (ECM). These factors, which include MMPs, are responsible for the eventual opening of the BBB, allowing infiltration of peripheral immune cells. BDNF and other neurotrophic factors are also scarce in the peri-infarct region, limiting the capacity for neural plasticity, and increased amounts of these factors may prove beneficial (George and Steinberg 2015).

Molecular-based treatments have been tried in an attempt to mitigate different mechanisms of cell death poststroke. Some drugs have been used to mitigate excitotoxicity that is mediated by GABA and N-methyl-D-aspartate (NMDA) receptors, while others have targeted the maintenance of Ca^{2+} homeostasis. Other strategies facilitate the transportation of various growth factors across the BBB, such as BDNF and glial-derived neurotrophic factor (GDNF), or to help maintain a more anti-inflammatory environment (Sekerdag et al. 2018).

While these treatments can undoubtedly help preserve cells and tissues after CNS trauma, the mechanisms that cause cell death are widely diverse and it would be challenging to develop a pharmacological solution to even multiple causes. While it is possible that solutions to certain aspects of the diseases can be solved through pharmacological means, an answer for a more comprehensive number of these problems likely lies in other therapeutic approaches.

Treatments for CNS Autoimmune Conditions

Much research on NMO has revolved around the interaction of AQP4-IgG with AQP4 receptors on the surface of astrocyte end feet, and the inflammatory cascade that follows depending on the complementary pathway. This disease state does not progress, such as MS and AD, but the diseases become severe as each inflammatory attack leaves further damage and demyelination in the CNS. For this reason, it is essential to control these attacks as much as possible, through both prevention and remedial means. Many of the strategies developed to treat this condition are geared to alleviate symptoms from acute attacks. One drug used in this approach is the corticosteroid methylprednisolone. Corticosteroids are well known to suppress inflammatory attacks by suppressing the circulation of circulating immune cells,

and the expression of adhesion molecules and matrix metalloproteinases (Papadopoulos et al. 2014). In cases where corticosteroids are not sufficient to alleviate the inflammation, therapeutic plasma exchanges are used to eliminate AQP4-IgG from the patient's circulation (Kleiter and Gold 2016). Other strategies also focus on the neutralization of AQP4-IgG. This can be done through the administration of tryptophan or protein A or through the injection of immunoglobulins.

Many of these treatments require further testing. Some, such as therapeutic plasma exchange, have established precedent in treating other autoimmune conditions such as multiple sclerosis, but examples of use for NMO have been more limited and there could be restrictions on patient eligibility (Kumar et al. 2018). While these treatments successfully treat autoimmune attacks, and as such are essential elements of treating NMO, they still seem to be only treatments of the symptoms and do not treat the underlying cause of the disease, highlighting the need for therapeutic strategies for NMO's underlying causes.

Prevention of NMO attacks has also been a large focus in recent research. Earlier approaches mirrored MS treatments, but some immunosuppressants used for MS were found to exacerbate NMO attacks. The use of immunosuppressants comes with an inherent risk of infections, and patients with certain chronic infections, such as HIV and hepatitis B and C, would be at high risk for these treatments. Additionally, pregnant women would likely be excluded from these preventative treatments (Huang et al. 2018; Kleiter and Gold 2016).

As the best option currently available to NMO patients, the use of immunosuppressants is unavailable to a large number of patients as preventative measures. These treatments, along with their secondary treatment methods, are often reactionary to an attack, after which irreversible damage may have already been done. There is still much research yet to be done on this condition. A better understanding of the mechanisms underlying these attacks could lead to the discovery of novel therapeutic strategies.

Unlike NMO, MS can develop into a progressive disease that becomes severe in later stages of development. However, the approach taken with current treatments is similar to that of NMO, where the focus is neutralizing acute attacks and prevention of future ones (Hauser and Cree 2020). As discussed above, the primary approach for the maintenance of MS is administering immunosuppressants to offset the effects of rampant inflammation seen in these attacks. Mechanistically, commonly used drugs are those which are disease-modifying. Some commonly used disease-modifying treatments are monoclonal antibodies, which bind to molecules like CD20 on B cells and α4β1 integrin, which help prevent antigen signaling by B cells and adhesion and infiltration of lymphocytes. The typical trend of disease-modifying approaches in MS is that they help with relapsing, but not progressive MS. The CD20-targeting antibodies bucked this trend and effectively treat both forms of MS, leading to the approval of ocrelizumab for both relapsing and progressive MS (Doshi and Chataway 2016; Hauser and Cree 2020). Other disease-modifying drugs target other mechanisms, such as induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to engage antioxidant function, as seen with

dimethyl fumarate, or the inhibition of activated lymphocyte proliferation, as seen with teriflunomide (Hauser and Cree 2020). As with any treatments, adverse side effects can be possible when using these therapies, and similar to NMO treatments, the use of immunosuppressants could lead to opportunistic infections. For example, one complication with ocrelizumab is the possible development of a severe herpes virus infection (Hauser and Cree 2020). One of the earlier drugs to be used, fingolimod, carries a risk of heart block upon initiation of treatment, and although rare, multiple bacterial and viral infections occur in patients to whom this drug is administered (Hauser and Cree 2020). While the best options to offset the relapsing nature of the disease in its early form, these disease-modifying treatments, with the notable exception of ocrelizumab, have had difficulty in sufficiently delaying and treating the onset of progressive MS (Feinstein et al. 2015; Hauser and Cree 2020). With limited options for the treatment of progressive MS, the field is still wide open for developing therapies that cater to the treatment of the advanced stage of this debilitating disease.

Treatments for Neurodegenerative Disorders

Treatment strategies for AD are limited, and while they generally improve cognition in patients, the rate of cognitive decline remains unchanged (Weller and Budson 2018). Cholinesterase inhibitors, such as donepezil, rivastigmine, and galantamine are considered go-to treatments for both AD dementia, as well as Parkinson's disease. Additionally, memantine, which is simultaneously an NMDA receptor antagonist and a dopamine agonist, is approved for AD patients with attention and alertness deficits (Weller and Budson 2018). Other preventative treatments have been presented, but their reliability remains in question. For example, huperzine A, isolated from a Chinese herb, has gained attention as a potential treatment to improve cognitive function for AD patients and is approved in countries outside of the USA. Recent systematic reviews have raised issues with how the studies on this therapeutic have been conducted, citing the potential for experimental bias and lack of long-term safety studies (Laver et al. 2016; Yang et al. 2013). Other preventative measures such as Vitamin D and omega-3 fatty acid supplements, and the use of nonsteroidal anti-inflammatory drugs (NSAIDs), have been put forward with some evidence of efficacy, but definitive proof has yet to be presented that these therapeutic strategies work (Weller and Budson 2018).

More recently, developing therapies have been targeting tau protein and the amyloid plaques. These strategies differ very much in their mechanistic approaches. Accumulation of amyloid plaques induces phosphorylation of tau proteins, so the targeting of amyloid plaques focuses on the underlying pathophysiology of the disease. In contrast, the targeting of tau proteins puts focus on what is believed to be the direct cause of the symptoms (Doggrell 2019). Approaches based on this hypothesis, dubbed the amyloid cascade, have overall made some progress in clinical trials, but definitive results remain elusive to demonstrate their efficacy. For example, the beta-secretase 1 inhibitor verubecestat showed great promise in

early animal and clinical trials, but failed phase III clinical trials, leaving very little progress in treatment by preventing amyloid plaque deposition (Doggrell 2019). Tau vaccines have also gained traction as an experimental approach, and while some have progressed to phase III clinical trials, it remains to be seen what kind of efficacy these treatments will have for human patients (Weller and Budson 2018).

Since HD pathology is downstream of a mutation in the HTT gene, treatment for the condition is more challenging than other CNS disorders. Some treatment strategies focus on modulating the transcription of HTT genes in DNA. These include zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENS). The goal of these nucleases is to cause a double-strand break in the DNA at the desired location. Specific nucleotide sequences can then be delivered with the intent that they will act as a template for homology-directed DNA repair after the break occurs (Tabrizi et al. 2019). The advantages of this approach are that it offers a potential long-term treatment for HD after a single administration of the treatment. The drawbacks of this treatment strategy are that they are irreversible and the potential lack of specificity could lead to the disruption of nontarget genes. CRISPR and Cas9 use a similar approach but do not use a protein domain to recognize the desired nucleotide sequences (Tabrizi et al. 2019). Though these newer approaches also present much promise in HD treatments, they are still more unique fields of research and must be further developed for application in human patients.

Similarly, small interfering RNA (siRNA) and synthetic microRNA (miRNA) are being utilized to suppress the translation of huntingtin proteins. This approach has shown promise in preventing the development of the disease in mice, and no adverse reactions to lowered huntingtin protein levels have been observed in early primate research. This indicates that while efficacy is yet to be seen phenotypically with this approach, it is well tolerated by animal subjects so far. Long-term exposure to these RNAs can, however, instigate an immune response through increased interferon expression and toll-like receptor interactions, as the RNAs can resemble viral products. Additionally, liver toxicity has been observed using these treatments (Tabrizi et al. 2019).

Other strategies focus on alleviating symptoms that occur as HD progresses, such as chorea, cognitive decline, and dementia. Two drugs, tetrabenazine and, more recently, deutetrabenazine, are currently approved for the treatment of chorea. While tetrabenazine was associated with the development of depression and suicidal thoughts, early evidence suggests that deutetrabenazine is better tolerated by patients, but more time is required in the market before a complete safety profile can be compiled for the latter of the two drugs (Heo and Scott 2017; Wyant et al. 2017). Other antipsychotic drugs have been tried with mixed results, and while other targets have been investigated, such as NMDA receptors, there has been little progress in developing other therapies for chorea in HD (Wyant et al. 2017).

Treatment of cognitive and psychiatric difficulties associated with HD has proven difficult. Hypotheses regarding excitotoxicity as a possible mechanism imply memantine may be efficacious, but further testing is required. For psychiatric conditions such as suicidality and depression, selective serotonin reuptake inhibitors

(SSRI) are a primary recommendation but can be dangerous when combined with other drugs, including opiates (Bruggeman and O'Day 2020; Wyant et al. 2017). A plethora of other treatments are currently being investigated to treat both the psychiatric and cognitive implications of HD and to discuss even their broader categories would take pages to cover in this chapter, but a useful summary of developing therapies can be found in *GeneReviews* (Caron et al. 1993).

Cell-Based Therapies for CNS Disorders

With the difficulties that accompany pharmacological and genetic approaches to CNS treatments, cell-based therapies are becoming more popular as an alternative therapeutic strategy. One strategy that has gained some attention in research is manipulating immune cell polarization, including microglia in the CNS. One specific example is seen in stroke research. It is believed that controlling the polarization of microglia and manipulating their phenotype to an anti-inflammatory M2 state may help control the rampant inflammation seen poststroke (Hatakeyama et al. 2020). Progress on this treatment angle could lead to breakthroughs in other conditions, as uncontrolled inflammation seems to be almost universal in CNS conditions, whether they are autoimmune or neurodegenerative. The shift in focus on glial cells may also be an aspect of therapy extrapolated upon. Similar focuses can be found when evaluating the overall activity of microglia in MS (Guerrero and Sicotte 2020). The elimination of overactive immunity is a central strategy for the treatment of NMO, as hematopoietic stem cell transplantation aims to in a manner "reset" a patient's immune system (Ceglie et al. 2020). Hematopoietic stem cells taken from umbilical cord blood have also been shown to improve the health of dopaminergic neurons in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) model of Parkinson's disease in mice, and they contribute toward angiogenesis after hind limb ischemia (Das et al. 2009; Lu et al. 2013). Additionally, hematopoietic stem cells taken from umbilical cord blood have shown efficacy in treating animal models of diabetes and osteoporosis, and have shown promise in treating myocardial ischemia after ex vivo expansion (Aggarwal et al. 2012; Kanji et al. 2014; Lu et al. 2010). Another approach has been to influence the proliferation and differentiation of endogenous neural stem cells (NSC), as seen in AD research, using the rationale that neurogenesis is severely limited in AD and that this strategy may counteract that aspect of the disease (Cosacak et al. 2020).

Additionally, multiple studies involving the application of mesenchymal stem cells (MSCs) of various origins have begun clinical trials, with some treatments reaching phase II as of 2020 (Cummings et al. 2020). MSCs have become a popular experimental treatment as they provide multiple benefits, including potent anti-inflammatory capabilities as well as their ability to supply paracrine support through the secretion of growth factors (Mishra et al. 2020).

One downside to the use of many types of MSCs is that they can be difficult to access (Mushahary et al. 2018). For this reason, dental pulp–derived stem cells (DPSCs) have become popular in therapeutic research. These cells are isolated from

the dental pulp of third molars, which are broadly discarded as routine medical waste. The isolation process for these cells is straightforward, and they can be easily passaged many times while keeping their stem cell properties (Suchanek et al. 2007; Yamada et al. 2019). They have repeatedly shown multipotency and are able to differentiate along osteogenic, chondrogenic, adipogenic, and neurogenic lineages (Osathanon et al. 2014). Another benefit of DPSCs and their potential application in CNS disorders is their origin in the neural crest, implying increased compatibility with the CNS (Noda et al. 2019). They also have been shown to secrete various growth factors like vascular endothelial growth factor (VEGF), as well as neurotrophic factors such as BDNF and GDNF. Already, DPSCs have shown some promise in treating several disease states, including osteoporosis, diabetic wound healing, and arthritis (Kanji et al. 2021; Rolph et al. 2020).

These advantages come with the other standard advantages of MSCs, such as potent anti-inflammatory activity as well as antioxidant activity. Given the broad spectrum in which these cells are able to operate, they are attractive as a multifaceted approach to the conditions described above. Their ability to modulate inflammatory attacks gears most toward controlling the activity of local microglia, and the inflammatory activity of infiltrating immune cells. Simultaneously, they can suppress the levels of reactive oxygen species and the harm caused by these molecules, which are often produced by immune cells and astrocytes undergoing severe gliosis, seen in each of these conditions (Ullah et al. 2018; Zhou et al. 2020). While negating the negative effects seen from inflammation and ROS, these cells can also facilitate neuroprotective effects and, potentially, neuroregeneration (Tsutsui 2020; Varga and Gerber 2014). While these cells may be ineffective in treating the underlying cause of some diseases, such as the mutations present in HD, they may be more efficacious in treating a broader number of symptoms that would normally take multiple drugs to cover.

The main difficulties in applying stem cell transplants are both mechanisms of delivery and the viability of stem cells postinjection. In particular, the delivery through the BBB, as with many CNS treatments, will have to be facilitated. In some conditions, there are windows when the BBB has increased permeability, such as during one of the biphasic openings of the BBB after a stroke occurs, and neurodegenerative diseases appear to compromise BBB integrity (Boese et al. 2020). This, combined with the fact that DPSCs have demonstrated the ability to home in on injured sites in both in vitro and in vivo experiments, presents some early promise to develop effective delivery methods (Kiraly et al. 2011; Xiao et al. 2017). Should these challenges be overcome, the potential upside to this therapeutic strategy is substantial. As discussed in the introduction, much of the CNS research that has historically been performed, and many of the therapeutic strategies thus far, have focused on preserving neurons. This, of course, is an essential aspect of promoting long-term recovery, but the incorporation of glial cells into the recovery equation, as well as having access to a treatment that can support the healthy function of both neurons and glial cells with minimal risk of immune rejection, could prove invaluable, as the health and functions of each of these cells are inseparably intertwined with each other **(**Fig. 4**).**

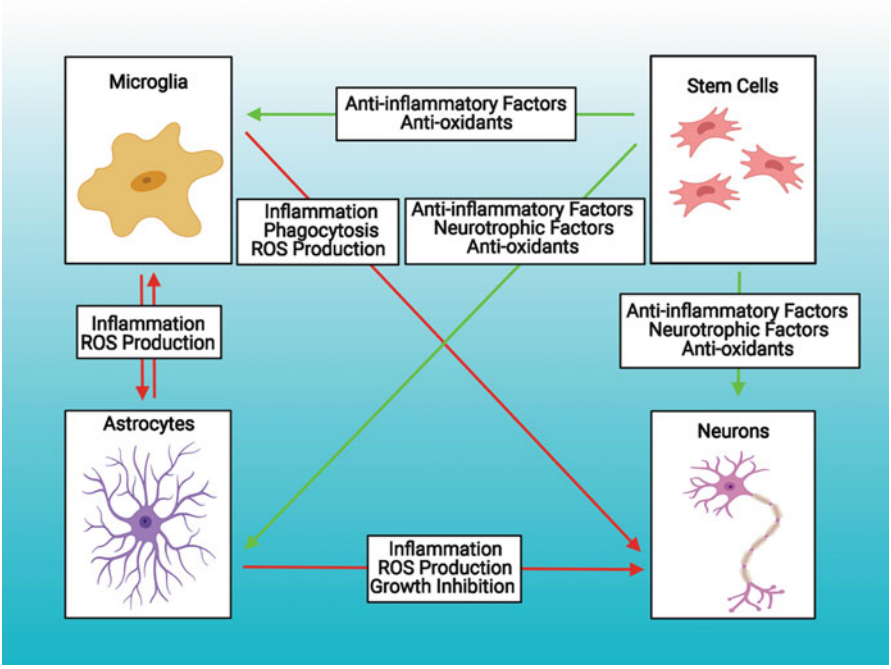

Fig. 4 DPSC effects on astrocyte and microglia functions and neuroprotection Astrocytes and microglia are both capable of producing inflammatory cytokines such as TNF- α and IL-1β. This leads to M1 activation in microglia, and can help induce gliosis in astrocytes. These signals also negatively impact neuronal function and can help facilitate the breakdown of synapses by microglia. ROS are also produced in both M1 microglia and reactive astrocytes. These cause a myriad of problems such as the oxidation and breakdown of cell membranes, which affects the function of all three cell types. DPSCs could help remedy these situations by secreting anti-inflammatory factors such as IL-10. They have also demonstrated anti-oxidant activity, counteracting ROS production from the glial cells. They are able to also provide neurotrophic support through the secretion of factors such as BDNF and GDNF, exerting a protective effect on both neurons and astrocytes

Conclusion

Research of the CNS and treatments of its pathologies have traditionally focused on neurons as the key to eliciting better outcomes in neuro-deficiencies and traumas. As research into the CNS has deepened, so has the arsenal of tools available to evaluate the roles of other cell types in both the physiology and pathologies of the CNS. These advances have been instrumental in revealing exactly how connected neurons and glial cell interactions are, and thus the importance not only of supporting neuronal health and regeneration but also of promoting the proper functions of microglia and astrocytes as supporting cells.

CNS pathologies are often multifaceted and difficult to treat with a single drug, and the drug approval process for many of these conditions, whether they are related

to CNS trauma, autoimmune mechanisms, or neurodegeneration, has proven to be challenging. While some treatments have been approved in the USA for these conditions, there remains a demand for more efficacious treatments that result in more favorable outcomes.

Viable alternatives are being researched to meet this need, despite the number of setbacks seen in the drug approval process. And while not without its challenges, the application of stem cell transplantation represents a field of great potential, as stem cells can act on multiple fronts and are able to react to their signaling microenvironment. The amount of potential that lies in these treatments remains to be seen, as this approach is still in its infancy and requires a large amount of optimization. As time passes, knowledge of the mechanisms underlying these pathologies will be better elucidated, and more effective therapies will follow. So, while there has been a steep uphill battle for treating CNS disorders, there is still room for optimism as the understanding of these diseases continues to grow and hopefully, in the near future, an effective regenerative therapy will be developed.

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Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs) as a Platform for Modeling Arrhythmias

29

Lisa Lin, Tiffany Barszczewski, Patrick G. Burgon, and Glen F. Tibbits

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Lisa Lin and Tiffany Barszczewski contributed equally with all other contributors.

L. Lin
Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada
e-mail: lla5@sfu.ca

T. Barszczewski · G. F. Tibbits (✉)
Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada

Cellular and Regenerative Medicine Centre, BC Children's Hospital Research Institute, Vancouver, BC, Canada
e-mail: tiffany_barszczewski@sfu.ca; tibbits@sfu.ca

P. G. Burgon (✉)
Department of Chemistry and Earth Sciences, College of Arts and Sciences, Qatar University, Doha, Qatar
e-mail: patrick.burgon@qu.edu.qa

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Abstract

Cardiac arrhythmias can arise due to a host of both genetic and acquired factors. Specifically, the genetic basis of arrhythmogenesis is not fully understood due to the lack of robust models that reliably recapitulate human physiology. Human-induced pluripotent stem cells (hiPSCs) have strengthened regenerative medicine by producing cells that bear the genetic signature of patients being studied. Upon differentiation into hiPSC-derived cardiomyocytes (hiPSC-CMs), these cells can be used to phenotype known mutations or suspected variants that may contribute to abnormal electrical activity in the heart. Furthermore, novel therapeutics can be screened for the management and treatment of arrhythmias in patient-specific hiPSC-CMs. In this chapter, we will briefly discuss the practical utility of hiPSC-CMs to study inherited arrhythmias with a specific focus on atrial fibrillation (AF), catecholaminergic polymorphic ventricular tachycardia (CPVT), and disruptive electrical events that may occur in patients with hypertrophic cardiomyopathy (HCM). We will describe an investigative pipeline that integrates genome editing, tissue engineering, biobanking, and systems biology as complementary approaches. Together, these various applications are directed toward a common goal of bench-to-bedside characterization of arrhythmias in patient-specific hiPSC-CMs.

Keywords

Arrhythmia · Cardiomyocytes · Cardiomyopathy · Fibrillation · hiPSCs · Pluripotent

Abbreviations

AF	Atrial fibrillation
AFM	Atomic force microscopy
CiPA	Comprehensive in vitro proarrhythmia assay
DCM	Dilated cardiomyopathy
ddPCR	Droplet digital polymerase chain reaction
ER	Endoplasmic reticulum
FFA	Free fatty acids
FFT	Fast Fourier transform
GECI	Genetically encoded calcium indicator
GEVI	Genetically encoded voltage indicator
GWAS	Genome-wide association studies
HCM	Hypertrophic cardiomyopathy
hESCs	Human embryonic stem cells
hiPSC-CMs	Human-induced pluripotent stem cell-derived cardiomyocytes
hiPSCs	Human-induced pluripotent stem cells

LQTS	Long QT syndrome
LTCC	L-type calcium channel
MEA	Multielectrode array
M-MLV	Modified Moloney leukemia virus
MS	Mass spectrometry
PBMCs	Peripheral blood mononuclear cells
pegRNA	Prime editing guide RNA
PTM	Posttranslational modification
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RyR	Ryanodine receptor
SCD	Sudden cardiac death
SNP	Single nucleotide polymorphism
SR	Sarcoplasmic reticulum
TEM	Transmission electron microscopy
TTs	T-tubules
VF	Ventricular fibrillation

Introduction

Human-induced pluripotent stem cells (hiPSCs) continue to enhance the study of complex diseases – especially those that contribute to certain cardiac pathophysiology – by putting personalized medicine within arm's reach. With this progress, researchers now have unprecedented access to cell-type and patient-specific assays, including high-throughput drug screening and disease modeling. Shinya Yamanaka was awarded the 2012 Nobel Prize in Physiology or Medicine for his team's transformative work in 2007, generating pluripotent stem cells from reprogrammed somatic cells (Takahashi et al. 2007). The hiPSCs share similar pluripotency and differentiation capacities to human embryonic stem cells (hESCs). The initial use of somatic cells circumvents the ethical challenges of using hESCs and are easily obtained through minimally invasive procedures (e.g., skin punch biopsies, blood draws, and buccal swabs). hiPSC lines can be maintained in culture endlessly and cryopreserved for long-term study.

Since Yamanaka's seminal publication, tremendous effort has been made to facilitate the differentiation of hESCs and hiPSCs into functional cardiomyocytes (Burridge et al. 2014; Lian et al. 2013). The process employs a chemically defined small molecule protocol to initially direct the hiPSCs toward a mesodermal lineage by adding a glycogen synthase kinase-3 (GSK-3) inhibitor. The subsequent cardiac specification is induced by a Wnt pathway inhibitor to produce spontaneously beating, primarily ventricular, hiPSC-CMs. Other cardiac cell types, namely, atrial and nodal hiPSC-CMs, can also be specified by the temporal addition of retinoic acid during the differentiation process (Protze et al. 2017).

With current advancements in bench-to-bedside research, hiPSCs from patients harboring pathogenic mutants (or suspect variants identified by genome-wide association studies (GWAS)) can be generated and differentiated into the hiPSC-CMs to

model heart rhythm conditions (Cagavi et al. 2018). To put the importance of this research field into perspective, an estimated three million individuals in the United States are affected by atrial fibrillation (AF). With a rapidly aging population, the number is expected to rise to upwards of 12 million by 2030 (Colilla et al. 2013; Miyasaka et al. 2006). In parallel, ventricular arrhythmias lapse into high incidences of hospitalization and may be responsible for up to 400,000 sudden cardiac death (SCD) cases each year (Khurshid et al. 2018; Roberts-Thomson et al. 2011). Inherited arrhythmias are a subset of these electrical events and are arguably more sinister in nature; by which an individual may experience ventricular tachycardia once in their life due to physical trauma or a myocardial infarct, patients who are genetically predisposed to an arrhythmic condition may face obstacles operating against their health daily. This predisposition can dramatically decrease the quality of life for individuals who may also be required to compromise activities such as exercise and play for extra caution.

The genetic and mechanistic bases of inherited rhythmic disorders are complicated, to say the least, although strategies to link gene variants to cardiac disease-associated arrhythmias have been ongoing for more than 30 years. Thus, an interdisciplinary approach (descendant from systems biology) using hiPSC-CMs in vitro is required to derive a mechanistic explanation for a given patient's arrhythmogenic phenotype in vivo. Furthermore, when a variant is identified in the patient's genome, a comprehensive characterization of that variant should be carried out.

In this chapter, we briefly describe a pipeline that consolidates hiPSC generation from patients in the clinic. The strategy includes quality control steps to establish cell lines for further hiPSC-CM differentiation. The hiPSC-CMs are then matured and cultured as 2D monolayers or 3D tissue, followed by phenotypic assays that describe the mutant cell line at a transcriptomic-proteomic and functional level (Fig. 1). Fundamentally, the techniques described will streamline characterization of inherited arrhythmias in patient-specific hiPSC-CMs from bench-to-bedside.

Establishing Patient-Derived hiPSC Lines

hiPSC Reprogramming

Retroviral transduction of four transcription factors (Oct3/4, Klf4, Sox2, and c-Myc), now colloquially coined the "Yamanaka factors," was shown to reprogram mouse embryonic and human adult fibroblasts into an embryonic stem cell-like state (Takahashi and Yamanaka 2006). The group then reprogrammed human adult dermal fibroblasts from skin punch biopsies with the Yamanaka factors into hiPSCs (Takahashi et al. 2007). Seki, Yuasa, and Fukuda (2012) developed a protocol to generate hiPSCs from terminally differentiated T cells in a small blood sample. Together with a less invasive approach to obtain patient cells, they also delivered the Yamanaka factors into the cells using a mutant Sendai virus that, unlike integrative retroviruses, could replicate within the cytoplasm and subsequently is destroyed (Seki et al. 2012). Additionally, non-viral and vector-free delivery of these

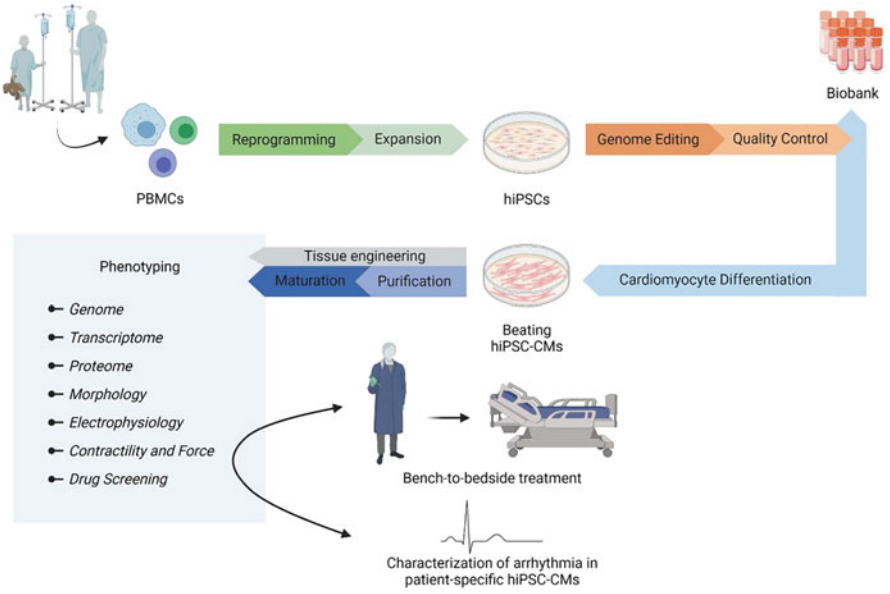

Fig. 1 Pipeline for the use of hiPSC-CM. *Genome edited hiPSCs that have undergone Q/C tests

transcription factors is recommended to mitigate unwanted tumorigenesis (Malik and Rao 2013; Narsinh et al. 2011).

hiPSC Quality Control and Genotyping

Careful consideration must be given to the viability, fidelity, and stability of patient (and any commercially obtained) hiPSC lines cultivated in vitro. The hiPSCs are taken through quality control (QC) processes after being generated from either peripheral blood mononuclear cells (PBMCs) or dermal fibroblasts. QC steps can include, but are not limited to:

- Genetic stability – Assessment of genetic stability by karyotyping to ensure 23 pairs of chromosomes and/or quantitative PCR (qPCR) detection of common karyotypic abnormalities (e.g., hPSC Genetic Analysis Kit, STEMCELL Technologies, Vancouver, Canada).
- Sterility – Routine mycoplasma detection methods are PCR-based mainly due to easy sample preparation for rapid mycoplasma screening (e.g., Mycoplasma PCR Detection Kit, Applied Biological Materials Inc., Richmond, Canada). Bacterial, fungal, and viral testing are also recommended.
- Pluripotency – Validation of hiPSCs' differentiation into ecto-, meso-, and endodermal germ layers (e.g., STEMdiff Trilineage Differentiation Kit, STEM Technologies, Vancouver, Canada). Alternatively, an in-house qPCR or droplet digital

PCR (ddPCR) assay can screen for relative (to housekeeping genes) or absolute expression, respectively, of pluripotency markers, such as Nanog (intracellular), Oct4 (intracellular), and TRA-1 (extracellular) transcripts.

Genotypic analysis of the patient cell line should be conducted, first by screening the genome against a panel designed for a specific disease as deemed by clinicians and researchers. The genome may then be studied further either by whole-exome sequencing or whole-genome sequencing, in combination with data from GWAS (Behr et al. 2013; Milan et al. 2010; Tucker and Ellinor 2014). The patient may have single nucleotide polymorphisms (SNPs) that are above a given P-value significance threshold identified in GWAS that may be associated with a particular disease phenotype; the first example of this SNP identification in AF was rs2200733 in chromosome 4q25 associated with AF (OR $\geq$ 1.6) (Gudbjartsson et al. 2007). In addition, sporadic or familial studies may require exon sequencing for genes encoding specific sarcomeric elements or ion channels to find amino acid changes that may affect protein interactions or structure (Dewar et al. 2017; Ellinor et al. 2010; Roston et al. 2018; Shafaattalab et al. 2019a). At this stage, the hiPSCs can be deposited in a biobank for other researchers worldwide to access for study, particularly if there are specific genetic variants, diagnoses, or arrhythmic patient phenotypes of interest.

Genome Editing Applications

Genome editing is a powerful tool for modeling arrhythmias and disease in hiPSC-CMs (Anzalone et al. 2019; Doudna and Charpentier 2014; Rees et al. 2019). With CRISPR/Cas9 genome editing, a homology-directed repair can facilitate precise base pair changes, as well as large insertions, using an exogenous DNA template (Ran et al. 2013). This technique is relatively straightforward, however; the editing efficiency in hiPSCs and other cell types remains inconveniently low (<3%). Some groups have taken the CRISPR/Cas9 system to new heights with modifications that increase editing efficiencies several fold and eliminate the necessity for double-strand breaks prone to insertions and deletions in non-homologous end-joining repair. Base and prime editing are two CRISPR-based genome editing techniques developed in the Liu lab at the Broad Institute of MIT and Harvard University; base editing utilizes a cytidine or adenine deaminase complexed to a Cas9 nickase to convert cytosine bases to thymine via a uracil intermediate or adenine bases to guanine via an inosine intermediate (Gaudelli et al. 2017; Komor et al. 2016). Prime editing has been making headlines due to the sophisticated design of the system and drastically improved editing efficiencies compared to traditional CRISPR/Cas9 (Anzalone et al. 2019). The protocol involves a Cas9 nickase, but this time fused to a modified Moloney leukemia virus (M-MLV) reverse transcriptase. A prime editing guide RNA (pegRNA) mediates both specific annealing to a target region of interest on the DNA strand, as well as a template sequence that the reverse transcriptase uses to add nucleotides along with the nick (Anzalone et al. 2019). The system is precise and can mediate all 12 point mutations, and smaller insertions and

substitutions up to 40 bp long (Anzalone et al. 2019, 2020). Prime editing efficiencies rely heavily on the initial design; notably, the Olson lab at the University of Texas Southwestern pioneered one of the first studies to use prime editing in hiPSCs. They could achieve impressive 20–54% editing efficiencies for a two-nucleotide exon correction in the dystrophin gene (Chemello et al. 2021).

Using the genome editing tools described, there is the freedom to make almost any desired base pair changes and targeted insertions. Importantly, isogenic controls of a patient cell line can be established by correcting suspect genetic variants (Hoekstra et al. 2012). One can reliably compare patient hiPSC-CM assay results with a control line matching their genetic signature by establishing a "corrected" version of their genome. To further investigate a potentially arrhythmogenic variant across a diverse range of genomes, variants of interest can be introduced into "wild-type" cell lines. These hiPSC lines can be procured from phenotypically healthy individuals, as well as commercial sources. Fluorescent tags like genetically encoded voltage indicators (GEVIs) and genetically encoded calcium indicators (GECIs) can also be used during live-cell imaging to track the electrical changes across the cell membrane to indicate action potential activity and morphology (Broyles et al. 2018; Shinnawi et al. 2015). The generation of GFP-sarcomere reporter lines can allow for direct contractility analysis using fast Fourier Transforms (FFTs) or similar tracking applications for further characterization of arrhythmias and associated cardiomyopathies (Sharma et al. 2018; Toepfer et al. 2019). Applications using these fluorescent tags, as well as establishing mutant and isogenic patient-specific hiPSC lines, are crucial in dissecting inherited arrhythmias and will be discussed later in this chapter.

hiPSC-CM Differentiation, Purification, and Maturation

Differentiation and Definition of Homogenous Cardiomyocyte Subpopulations

Protocols for hiPSC-CMs differentiation generally involve temporal modulation of the canonical Wnt/β-catenin signaling pathway by the addition of small molecules in defined culture media (Burridge et al. 2014; Lian et al. 2013) (Fig. 2). The previously described protocols typically result in 60–85% ventricular-like, 5–30% atrial-like, and 3–15% nodal-like hiPSC-CMs subpopulations with the remainder of the population containing fibroblasts or other non-cardiac cells (Devalla et al. 2015; Hoekstra et al. 2012; Itzhaki et al. 2011; Laksman et al. 2017; Protze et al. 2017). Using more specific cardiac cell types may be useful depending on the arrhythmia being investigated. For example, the retinoic acid signalling pathway has been shown as a regulator of both atrial and nodal lineages. Thus an increase in the percent population of atrial cells and nodal cells can be achieved with the timed addition of retinoic acid during the differentiation process (Gunawan et al. 2021; Lian et al. 2013; Protze et al. 2017). Other strategies to generate chamber-specific cells have also used directed

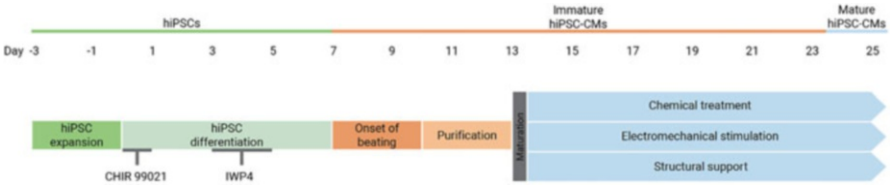

Fig. 2 Directed differentiation of hiPSCs into immature, fetal-like hiPSC-CMs. Following population purification, chemical, electromechanical, and/or structural treatments are used to induce maturation to produce hiPSC-CMs with more adult-like phenotypes

differentiation protocols combined with electrical conditioning to create electrophysiologically distinct atrial and ventricular tissue (Zhao et al. 2019).

The cardiomyocyte population can be further purified from non-hiPSC-CM cells, which may be vital to avoid confounding data from these cells in the phenotypic assays described later in the chapter (Fig. 2). Enrichment of the cardiomyocyte cell population can be achieved through cardiomyocyte-specific antibody selection combined with a magnetic column (e.g., PSC-Derived Cardiomyocyte Isolation Kit, Miltenyi Biotec, Auburn, CA, USA). Alternatively, metabolic selection, which employs a change from regular maintenance media to glucose-depleted and supplemented with sodium L-lactate, can be used to eliminate non-cardiomyocyte cell types (Feyen et al. 2020). The principle behind this strategy is that cardiomyocytes, unlike most other cell types, preferentially use free fatty acids (FFAs) as a substrate for energy consumption but can use other substrates as well. In the absence of glucose, CMs are still able to efficiently produce energy from lactate and survive (Tohyama et al. 2013). However, non-cardiomyocyte viability is low with a multi-day treatment of this metabolic selection media, and thus, they are eliminated from the culture. It is important to note that the co-culture of cardiac fibroblasts or endothelial cells is a promising strategy for structural and electrophysiological hiPSC-CM maturation (Beauchamp et al. 2020; Kim et al. 2010b). Thus, these non-cardiomyocytes may need to be cultured separately and mixed with a specific hiPSC-CM population in a defined quantity. With near 100% hiPSC-CM subtype specification, modeling arrhythmias and therapeutic drug screening are more refined for further translation to in vivo patient cell phenotypes.

Maturation

Maturation of the hiPSC-CMs remains one of the most significant barriers to their application in research and clinical therapeutics. While they are still able to recapitulate many arrhythmogenic phenotypes observed in patients and other models (e.g., murine models, biochemical studies), hiPSC-CMs produced under standard differentiation protocols retain many of the structural and functional qualities of a fetal cardiomyocyte. Current research is focused on tackling the challenge of simulating the cardiomyocyte maturation process in vitro (Karbassi et al. 2020; Lundy et al. 2013;

Marchianò et al. 2019; Piccini et al. 2015; Sun and Nunes 2017), though complicated and not fully achieved yet. Many strategies for hiPSC-CM maturation are outlined in the literature and encompass guided electrical, chemical, and metabolic treatments (Feyen et al. 2020; Garbern et al. 2020; Ronaldson-Bouchard et al. 2018; Sun and Nunes 2017). In addition, the growth of hiPSC-CMs as 3D tissue, such as organoids, spheroids, or microtissue mounted on structural scaffolding, has helped to enhance some phenotypes that are missing in immature 2D tissue, including the development of T-tubules (TT) for excitation-contraction coupling (Kim et al. 2010a; Parikh et al. 2017; Ronaldson-Bouchard et al. 2018; Sun and Nunes 2017). Integration of these approaches, which involve mechanochemical and metabolic cues, could help activate multiple, cascading molecular signalling pathways to enact developmental changes in the cells ranging from transcriptional and protein expression to tissue and organ morphology and function (Fig. 2).

Phenotypic Assays

Transcriptomic and Proteomic Analysis

Most studies of this nature employ transcriptional analyses such as real-time quantitative PCR (RT-qPCR) to give insight into the average expression profile relative to housekeeping genes of the hiPSCs or hiPSC-CMs. However, newer techniques like ddPCR, Nanostring, and bulk- and single-cell RNA-seq can capture a high-resolution, high-throughput snapshot of transcript heterogeneity within a cell population (Geiss et al. 2008; Paik et al. 2020; Taylor et al. 2017). Transcriptomics can be applied to quantifying the expression of markers for pluripotency, cell subpopulations post-differentiation, and markedly, cardiac maturation. In the context of modeling arrhythmias, transcript expression of genes associated with intracellular Ca^{2+} handling, ion channel expression, contractile function, and gap junction coupling between hiPSC-CMs can be assessed (Guo et al. 2019; Kamdar et al. 2020). While transcriptional analysis is useful, RNA expression levels often do not reflect protein concentrations due to differential rates of transcription and translation or protein trafficking within the cell. Different stressors and physiological demands also affect RNA and protein expression levels; thus, protein expression analysis should be integrated when phenotyping arrhythmias in hiPSC-CMs. Protein characterization is traditionally conducted using SDS-PAGE and western blotting, however; mass spectrometry (MS)-based proteomics provides a more detailed, more quantitative, non-biased, and meticulous account of protein up or downregulation, isoform distinction, and posttranslational modifications (PTMs). Bottom-up MS proteomics is a high-throughput method of quantifying global protein regulation by digestion of proteins into peptides. Alternatively, a top-down approach of MS-based proteomics is recommended by Cai et al. (2019) in hiPSC-CMs for increased specificity of protein targeting. Compared to bottom-up proteomics, the proteins are kept intact for full sequence coverage, allowing for more refined isoform and PTM identification (Cai et al. 2019). Top-down proteomics has been used to assess hiPSC-CM

maturation markers (Cai et al. 2019) and characterize proteomic changes in explanted septal tissue from HCM patients, including PTMs such as phosphorylation of the troponin complex subunits (Tucholski et al. 2020). Together with transcriptomic analysis in hiPSC-CMs, signaling pathways and molecular changes in inherited arrhythmias can be linked to the clinical manifestation of the disease.

Metabolic Considerations

One of the critical markers of cardiomyocyte maturation is the metabolic switch from glycolysis to fatty acid oxidation, occurring during fetal development (Batho et al. 2020; Hu et al. 2018; Lin et al. 2017; Nakano et al. 2017). This transition is coupled to producing many sarcomere-arrayed mitochondria for high oxidative phosphorylation capacity and energy yield from free fatty acids (Piquereau and Ventura-Clapier 2018). Media containing FFAs and other chemical substrates can be applied to hiPSC-CMs to facilitate a switch in their metabolism, which is largely glycolytic (Feyen et al. 2020). There is also a hypothesis that sarcomeric HCM mutations contribute to poor ATP use and thus energy depletion, which may be further explored (Ashrafian et al. 2003). Changes in hiPSC-CMs' energetics can be assessed using a Seahorse Analyzer assay (Agilent Technologies, Santa Clara, CA, USA), yielding critical information about the metabolic state of the hiPSC-CMs (Feyen et al. 2020). In this assay, a calibrated sensor near the cell surface detects changes in both the pH and the O_2 concentration in the surrounding media. These analyses provide a measure of cellular processes such as the oxygen consumption rate (OCR; a measure of cellular and mitochondrial respiration over time reported in picomole/minute), extracellular acidification rate (ECAR; a measure of proton extrusion into the extracellular medium over time reported in mili-pH/min), and proton efflux rate (PER; a measure of extracellular acidification accounting for media buffering capacity and plate geometry over time, reported in picomole/minute). These data give insight into the metabolic state of the cells and can be correlated with expression and morphological changes in mitochondrial size, quantity, development, and organization.

hiPSC-CM Morphology

Morphological analysis is a powerful aspect of hiPSC-CM phenotyping to identify biological perturbations, such as gap junction uncoupling and loss of TTs, which may contribute to electrical disarray and arrhythmias. Transmission electron microscopy (TEM) offers the highest resolution view of cellular ultrastructure as detailed as the dyadic coupling of L-type calcium channels (LTCC) and ryanodine receptors (RyRs) at the sarcoplasmic reticulum (SR). Both cardiomyocyte development and dysfunction can often be linked to pathological changes at the ultrastructural level. Despite being a low-throughput technique, TEM data offers a wealth of information about the state of the cell. For a global assessment of cell and tissue morphology, a

method from the Carpenter lab may be used. Cell painting involves multiplexing several fluorescent dyes and/or antibodies (such as those used in immunocytochemistry) to produce a high-throughput morphology screening tool (Bray et al. 2016). Visual readouts from each detection channel can allow extraction and analysis of 1500+ features, including shape, texture, and spatial relationships between stained (Bray et al. 2016). Morphological profiling with cell painting can also be used to assess the efficacy of hiPSC-CMs development and maturation, as well as the acute or chronic effects of certain drug treatments on labeled cellular structures. Hints as to the mechanism of action of an arrhythmia may be revealed in an unbiased manner based on observed morphological changes.

Contractility and Force Generation

The ability of cardiomyocytes to generate force is dependent on several factors, including the structural alignment of the sarcomeres, cytosolic Ca^{2+} handling, temperature, and drug effects. The physiological mechanisms of contractility are reasonably well understood (Chapman 1983; Kobayashi and Solaro 2005). However, disease mechanisms for contractility-related diseases, such as hypertrophic cardiomyopathy, are still not well understood. Although there are several theories as to the mechanisms of dysfunction, more research is necessary to determine how and why Ca^{2+} mishandling results in structural remodeling and arrhythmogenesis. Various aspects of contractile properties can be measured through several techniques encompassing FFT analysis, edge detection, and impedance-based microarrays. An important, high-throughput MATLAB software called SarcTrack can individually track fluorescently labeled sarcomeres in hiPSC-CMs and assess sarcomere content, beat rate, and calculate the rate of contraction and relaxation (Toepfer et al. 2019). In addition, atomic force microscopy (AFM), can also be used to determine the stiffness and force of contraction of the contractile unit (Borin et al. 2018; Chang et al. 2013). Evaluation of these functional properties of the hiPSC-CMs would provide critical information about disease states and provide a means to test how the cells respond to pharmacological agents that carry cardiotoxic risks.

Electrophysiological Measurements and Arrhythmia Assessment

Cardiomyocyte function is greatly dependent on the electrophysiological properties of cells at the single-cell level or as part of a functional syncytium. Three main techniques can be used to assess electrophysiology. Microelectrode array (MEA) systems, like the Maestro Pro multi-well MEA and impedance system (Axion Biosystems, Atlanta, GA, USA), serves to assess electrical activity and contractility in response to perturbations like drug additions or pH changes. Electrodes embedded in each well provide a method for applying electrical stimulation to the cells and recording field potentials across the tissue layer. Changes in hiPSC-CM electrophysiology can be continuously observed over days or weeks due to the method's non-invasive approach.

Optical mapping is an imaging technique that uses dyes or genetically encoded reporters (e.g., GECIs) for the determination of membrane voltage (e.g., RH-237, Fluovolt) and Ca^{2+} (e.g., Rhod2-AM, Fluo-4) (Lin et al. 2015; Shafaattalab et al. 2019b). This technique can provide high spatio-temporal resolution across a tissue that can then be used to access action potential morphology, propagation across tissue, and Ca^{2+} handling in response to arrhythmogenic triggers such as beta-adrenergic stimulation, increase packing rate, or arrhythmogenic drugs. Light-gated ion channels can be used to either stimulate or quiesce (e.g., BLINK 2) cell movement during imaging. The latter can be used to mitigate movement artifacts during live-cell imaging, without using drugs like blebbistatin, a commonly used myosin II inhibitor, or para-aminoblebbistatin, a photostable and non-fluorescent derivative of blebbistatin (Alberio et al. 2018).

Patch clamping, in many respects, is the "gold standard" of electrophysiological analyses and is a technique that allows one to measure either ion currents (voltage clamp) or membrane potential (current clamp) in single living cells. This technique is the only one of the three mentioned in this chapter that can be used to measure the absolute membrane potential (in mV) or ionic current densities (mA/pF). It is also the only means of examining the impact of genetic variants on ion channel biophysics. Patch clamping allows for the most accurate evaluation of the relative contribution of various ion channels to the cardiac action potential. However, there are disadvantages as well. First, to voltage clamp a cell, it is necessary to have a space clamp. This requirement is not possible in a functional syncytium as the cell-to-cell coupling allows the spread of electrotonic currents between cells. Thus, enzymatic (e.g., collagenase) cell dissociation is needed, which in itself can change cellular properties. Furthermore, arrhythmias can only be genuinely studied at a tissue (i.e., multicellular) level of organization. Thus, there are limitations to the conclusions one can make from examining a single cell. Secondly, this approach is the most technically challenging compared to others described here, both in data acquisition and data analysis. Thirdly, patch clamping is very labor intensive; the number of cells that can be examined is very limited and, therefore, prone to sampling errors. The second and third limitations may be mitigated somewhat by automated patch clamping instruments (e.g., Nanion PatchLiner, Sophion QPatch II), but they tend to be expensive.

In sum, all three of these techniques can be used to study cardiac electrophysiology, and each provides a different set of information that can be applied based on the requirements of the research question.

hiPSC-CMs for Proarrhythmic Drug Assessment

Electrical phenotypes may be unique on a case-by-case basis, and consequently, patients require extensive assessment and diagnosis that can only be enhanced by personalized medicine. Antiarrhythmic drug efficacy can be low and sometimes actually proarrhythmic. Nearly 90% of pharmacological treatments that have shown promise in research settings exhibit poor outcomes in Phase III clinical trials

(Colatsky et al. 2016). Additionally, non-cardiac-related drugs have often been pulled after trial testing due to unexpectedly causing arrhythmias in test subjects. This discrepancy is often due to key physiological differences between the animal models used in the research setting compared to humans resulting in poor clinical translation. Thus, an initiative called the Comprehensive in vitro Proarrhythmia Assay (CiPA) was created to screen proarrhythmic risk of pre-clinical drugs (Colatsky et al. 2016). Because clinical trials are expensive, it is crucial that potential pharmacological interventions be tested for efficacy and cardiotoxicity in vitro. Using hiPSC-CMs, multiple drugs can be screened in parallel at no risk to the health of the patient. Thus, hiPSC-CMs present an attractive model for drug testing for pro-arrhythmic risk as they can model a given patient's genotype and phenotype.

Conclusion

Employing a systems biology approach allows investigators to corroborate electrical dysfunction in patients across many interdependent disciplines. Increased prevalence of hiPSCs in preclinical screening should provide significant insight to our knowledge of inherited arrhythmias that can be leveraged toward developing new therapeutic strategies. Furthermore, the capacity to model patient-specific genetic backgrounds allows one to observe patient-specific responses to different treatment approaches, thereby identifying reproducible pathophysiological characteristics as suitable drug targets for novel therapeutic interventions. Many studies to date have used hiPSC-CMs along a similar pipeline as described in this chapter to study arrhythmias, such as long QT syndrome (LQTS) (Moretti et al. 2010; Cagavi et al. 2018), hypertrophic cardiomyopathy (HCM) (Lan et al. 2013), dilated cardiomyopathy (DCM) (Sun et al. 2012), Brugada syndrome (Nijak et al. 2021), SCDs (Shafaattalab et al. 2019a), atrial fibrillation (AF) (Ahlberg et al. 2018; Benzoni et al. 2020; Laksman et al. 2017), and arrhythmogenic RV dysplasia (Khudiakov et al. 2017). In addition, the use of hiPSC-CMs in combination with novel biomaterials and tissue engineering technologies to enhance structural and functional development will enable a more comprehensive analysis of arrhythmic phenotypes and drug-induced functional changes in vitro. The extensive work to date highlights the array of technologies available to improve hiPSC-CMs development and maturation.

The focus must now be shifted toward integrating these technologies to produce physiologically and functionally accurate representations of adult myocardial tissue. However, adopting hiPSC-CMs tissue engineering strategies for improved disease modeling will require the development of uniform procedures capable of addressing issues such as cardiomyocyte maturation and effective recapitulation of disease states. The various applications described are directed toward a common goal of bench-to-bedside characterization of arrhythmias in patient-specific hiPSC-CMs. High-throughput, disease-specific assays screened against focused libraries may allow for the discovery of candidate therapeutic targets through the interrogation

of the entire transcriptome and proteome, thus resulting in a powerful paradigm shift in drug discovery and therapeutic strategies.

Cross-References

- ▶ Advances, Opportunities, and Challenges in Stem Cell-Based Therapy
- ▶ Bioengineering Technique Progress of Direct Cardiac Reprogramming
- ▶ Current State of Stem Cell Therapy for Heart Diseases
- ▶ Induced Pluripotent Stem Cells
- ▶ Molecular Signature of Stem Cells Undergoing Cardiomyogenic Differentiation
- ▶ Stem Cell Applications in Cardiac Tissue Regeneration
- ▶ Therapeutic Uses of Stem Cells for Heart Failure: Hype or Hope
- ▶ Unraveling the Mystery of Regenerative Medicine in the Treatment of Heart Failure

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Induced Pluripotent Stem Cells

30

Progress toward Clinical Translation from Bench to Bedside

Adegbenro Omotuyi John Fakoya, Adekunle Ebenezer Omole, Nihal Satyadev, and Khawaja Husnain Haider

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Abstract

Induced pluripotent stem cells (iPSCs) offer huge promise and potential in the creation of patient-specific stem cells for modeling human diseases, drug development and testing, and personalized regenerative cell-based therapy. As we

A. O. J. Fakoya (✉) · N. Satyadev
Department of Anatomical Sciences, University of Medicine and Health Sciences, Basseterre, Saint Kitts and Nevis
e-mail: gbenrofakoya@gmail.com; nsatyadev@umhs-sk.net

A. E. Omole
Department of Anatomical sciences, American University of Antigua College of Medicine, St. John's, Antigua and Barbuda
e-mail: kunlesty@yahoo.com

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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journey into the second decade of their discovery, we investigate some of the hurdles limiting their use and the recent advances that have been made to address the limitations and to successfully move these cells from the bench to the bedside. This chapter discusses the progress made in theranostic applications of pluripotent stem cells during the last decade and a half and introduces the currently ongoing clinical trials that involve iPSCs to treat patients.

Keywords

Biobanking · Clinical trials · Disease modeling · Drug discovery · Heterogeneity · Immunogenicity · Induced pluripotent stem cells · Pluripotency · Stem cells · Tumorigenicity

Abbreviations

AMD	Age-related macular degeneration
CNV	Copy number variations
ESCs	Embryonic stem cells
hiPSCs	Human-induced pluripotent stem cells
HLA	Human leukocyte antigen
iPSC-RPE	iPSC-derived retinal pigment epithelial cells
iPSCs	Induced pluripotent stem cells
OOC	Organ on chip
RPE	Retinal pigment epithelial
SCA	Sickle cell anemia
SNV	Single nucleotide variation

Introduction

The pioneering work of Yamanaka and Takahashi led to the discovery of induced pluripotent stem cells (iPSCs) in 2006, which has turned out to be a significant breakthrough in stem cell research and for which they were awarded Nobel Prize in 2011 (Takahashi and Yamanaka 2006). Shinya Yamanaka and his team described the reprogramming of skin fibroblasts from mouse to pluripotent stem cells by viral induction of a quartet of transcriptional factors, namely, Oct 3/4, Sox2, Klf4, and c-Myc (OSKM/Yamanaka factors) (Ibrahim et al. 2016). In 2007, Shinya Yamanaka and his team used the same reprogramming protocol to create human iPSCs (hiPSCs) from human somatic cells (Takahashi et al. 2007). Working independently from Yamanaka's team, in 2007, Dr. James Thomson's research group described the development of hiPSCs, employing a similar approach but using a different combination of transcription factors (Yu et al. 2007). Like embryonic stem cells (ESCs) for their differentiation potential (both spontaneous and in response to specific cues) (Heng et al. 2005; Rufaihah et al. 2007, 2010; Bai et al. 2015; Liu et al. 2018), iPSCs are considered surrogate ESCs with infinite proliferation in culture. They can differentiate into all the cell types from the three embryonic germ cell layers

(ectoderm, mesoderm, and endoderm). However, unlike ESCs, which are derived from preimplantation embryos and thus their generation and use are fraught with moral and ethical issues relating to embryo destruction (Martin 1981; Evans and Kaufman 1981; Omole and Fakoya 2018) and ▶ Chap. 39, "Common Ethical Considerations of Human-Induced Pluripotent Stem Cell Research," this volume, iPSCs certainly provide a renewable source of stem/progenitor cells without any moral or ethical issues, thus providing a valuable alternative to ESCs. Indeed, iPSC technology has successfully removed any roadblock for the advancement of pluripotent stem cell research and their onward clinical application (Omole and Fakoya 2018). Given their biological and functional similarity with ESCs, iPSCs offer immense theranostic promise and potential in creating patient-specific stem cells for human disease modeling, drug development and testing, and personalized regenerative cell-based therapy for both adult and pediatric applications (Omole and Fakoya 2018; Cagavi et al. 2018; Çetinkaya and Haider 2020). This chapter presents a concise review of iPSC research, its challenges and progress, and the current status of iPSC clinical application.

From Classical Transcription Factor Quartet Transduction to Small Molecule Manipulation Protocols

Since the inception of the protocol for somatic cell reprogramming to pluripotency using a quartet of pluripotency determinant transcription factors (Oct4, Sox2, Klf4, and c-Myc (OSKM)), various research groups have been involved in the optimization of protocols to ensure the safety of the reprogrammed cells for human use. Despite the diversity of the modified protocols, the pluripotent cells obtained have shown remarkable morphofunctional similarity with iPSCs generated through the classical protocol of Yamanaka and Takahashi. These protocols encompass approaches with a lesser number of transcriptional factors using viral and nonviral vectors for delivery, protein-based systems, use of small molecules, etc. (Huangfu et al. 2008; Cho et al. 2010; Pasha et al. 2011; Fusaki et al. 2009; Meissner et al. 2007; Park et al. 2008). The latest advancement in this regard is to treat the transcription-modified somatic cells with P334, a mycosporine-like amino acid that significantly increases the cell reprogramming efficiency of OKSM transduced tail fibroblasts (Yoo et al. 2020). Besides other factors, the choice of cells as a "starting material" for reprogramming, based on their genetic and epigenetic composition, remains a critical factor enhancing the efficiency of the protocols and ensuring the high quality of the iPSCs (Raab et al. 2014). Some of the promising cell types used include liver and stomach cells (Aoi et al. 2008), bone-marrow-derived mesenchymal stem cells (Buccini et al. 2012), human skeletal myoblasts (Ahmed et al. 2011a), endothelial progenitor cells from umbilical cord blood (Gao et al. 2021), peripheral blood (Sommer et al. 2012), lymphoblastoid cell lines (Barrett et al. 2014), etc. Researchers have also used chemical compounds like valproic acid, 5-azacytidine, Parnate, and vitamin C to improve the efficiency of iPSC generation and also to generate "chemical-only" induced iPSCs (Li et al. 2009;

Chung et al. 2010; Su et al. 2013). Conversely, compounds that inhibit the p53 pathways have been shown to raise the efficiency of reprogramming by 23-fold when compared to the use of OSKM factors alone (Hong et al. 2009). MicroRNA has been employed either alone or in combination with OSKM factors to improve the efficiency of the reprogramming protocol (Judson et al. 2009; Subramanyam et al. 2011). An overview of cell sources has been elegantly provided by Ray et al. in their recently published review of the literature (Ray et al. 2021).

Despite the dynamic epigenetic changes that ensue during reprogramming, it is believed that some of the epigenetic memory of the "mother cell" is carried forward in the derivative reprogrammed cells. In the light of these observations, we have successfully generated IPSCs from skeletal myoblasts and bone-marrow-derived mesenchymal stem cells, two of the cell types that have progressed from bench to bedside for myocardial repair and regeneration in human patients (Ahmed et al. 2011a; Buccini et al. 2012). When transplanted in experimental animal models of acute myocardial infarction, the cells underwent both vasculogenic and myogenic differentiation, resulting in increased vascular density and neomyogenesis and leading to preserved global cardiac function (Afzal et al. 2011).

Despite these encouraging data, the use of iPSCs is not without its limitations, some of which are more related to their heterogeneity and pluripotency. Therefore, it is imperative to address these limitations, and some remedial measures should be adopted to ensure their progress to clinics for safe and efficient use in the patients.

iPSCs' Limitations – Overcoming Barriers to Clinical Translation

As discussed in the previous section, there are many challenges associated with the classical reprogramming protocol of iPSC generation reported by Yamanaka and his team. The last decade and a half have witnessed remarkable progress and advancement made to the original method of iPSC generation, and these technical improvements have helped address many of these limitations. Here, we identify these limitations and briefly mention what has been done so far to overcome them.

Low-Efficiency Reprogramming of Somatic Cells

The efficiency of iPSC generation reported by Yamanaka and his team using their classical protocol was meager, as low as 1 out of 10,000 skin cells completing the reprogramming process and successfully forming iPSCs (Takahashi and Yamanaka 2006; Okita et al. 2007; Takahashi et al. 2007). Intriguingly, new molecules have now been discovered that enhanced both the reprogramming method and its efficiency (Omole and Fakoya 2018). It is pertinent to mention that the earlier concept that reprogramming is a spontaneous happening has been replaced by a more realistic understanding of the mechanism. It is now being considered a systematic process, which consists of an early or the deterministic phase, stochastic phase, and the late or deterministic phase. Moreover, each one of these phases is characterized

by certain specific changes in the somatic cells undergoing reprogramming, which collectively determine the success of the procedure (Buganim et al. 2013). The occurrence of various molecular events leading to switching on of cells' regulatory network of genes responsible for pluripotency have been elucidated and a direct comparison of these events in the human and mouse cells have been well explained by Tada's group (Teshigawara et al. 2017). Given the complexity of the events happening therein, it also necessitates the intricate manipulation of the cells such that the game of probability involved in the reprogramming process favors a more efficient outcome.

A further in-depth understanding of the molecular events during the reprogramming of somatic cells will be helpful in identifying the bottlenecks that will help alleviate these hurdles to enhance the rate of successful reprogramming and in the development of the iPSCs, which will be genetically more stable. Furthermore, the efficiency of reprogramming is also dependent on variable factors, such as the choice of donor cell types for use as a starting material, reprogramming factors, delivery vehicles and methods, and culture conditions for the cells during and after reprogramming (Omole and Fakoya 2018). The hope is that researchers can carefully exploit these variable factors to ensure an exponential increase in the efficiency of iPSC generation.

Tumorigenicity

Tumorigenicity remains a common feature among pluripotent cells, irrespective of their origin. For example, iPSCs developed from skeletal myoblasts using the classical four-factor transduction protocol were used to treat an infarcted heart in an experimental animal model of acute myocardial infarction. The authors observed that intramyocardial transplantation of iPSCs led to cardiac tumor formation in 33% of the animals treated with iPSCs, thus raising a serious concern regarding the use of undifferentiated iPSCs (Ahmed et al. 2011b). Similar reports have also been published from other research groups (Kanemura et al. 2014; Deng et al. 2018). The tumorigenic potential of iPSCs has been attributed to the involvement of diverse factors. For example, in the original report on iPSC development, tumor formation was observed in ~20% of the offspring from the adult chimera from seven iPSC clones (Okita et al. 2007). This was attributed to the reactivation and overexpression of c-Myc transgene. Similarly, there is also the risk of insertional mutagenesis from the use of virus-based delivery methods, which can lead to tumor formation (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007). These two risks are specific for iPSCs as they are related to the reprogramming factors and the delivery method used to introduce the factors into the somatic cells. Additionally, tumorigenicity can result from incorrect or incomplete patterning and genetic instability (Yoshihara et al. 2017b; Yamanaka 2020). Inaccurate or incomplete patterning refers to the retention of undifferentiated and immature cells in the final cell products that were differentiated from human pluripotent stem cells. Hence, the undifferentiated cells can form teratoma. The tumorigenicity risk from incomplete

patterning and genetic abnormalities is not unique to iPSCs. Still, it is common to all cells that need long-term in vitro expansion to achieve the sufficient number needed for cellular therapy.

Genetic alterations are pretty common during the reprogramming process, as reported by many research groups, including chromosomal aberrations, single nucleotide mutations, copy number variations, etc. (Turinetto et al. 2017; Yoshihara et al. 2017a). This genetic alteration may already exist in the primary somatic cells, or they may develop during the reprogramming process (Yoshihara et al. 2017b). The first clinical study involving hiPSCs in 2014 was temporarily halted following the discovery of mutations in the hiPSCs belonging to the second patient, although they were not found in the primary somatic cells (Kimbrel and Lanza 2015; Attwood and Edel 2019).

Stem cell scientists have since addressed some of these contributory factors, which are somehow considered to be responsible for the tumorigenic potential of iPSCs. For example, mechanistic studies have demonstrated that c-Myc is dispensable for the successful reprogramming of the cells to pluripotency (Nakagawa et al. 2008; El Khatib et al. 2016). Interestingly, James Thomson's group generated their iPSCs using a distinct set of four reprogramming factors – Oct 3/4, Sox2, Nanog, and Lin 28 (OSNL) – and excluded c-Myc to prove its less significance and, hence, dispensability in the process (Yu et al. 2007). Nonviral delivery methods, such as plasmid vectors and transposons, and nonintegrative delivery methods, such as Sendai and adenovirus, have all been developed to eliminate the problem of insertional mutagenesis (Deng et al. 2015; Kang et al. 2015; Omole and Fakoya 2018). With regard to incorrect patterning, researchers attempting to bring iPSCs directly from the bench to the clinics must develop means to prevent tumor formation so they can meet the safety standards required for clinical trials. In order to avoid the issue of tumorigenicity, there have been attempts to directly reprogram the somatic cells to the lineage of interest without achieving a pluripotency status (Ahmed et al. 2012).

A brief look into some of the ongoing clinical trials involving iPSCs reveals some of the purification methods used to prevent incorrect or incomplete patterning. Such strategies include efficient directed differentiation protocols and positive/negative selection markers using antibody cell sorting systems like fluorescence-activated cell sorting and magnetic-activated cell sorting (Riordon and Boheler 2018; Simonson et al. 2015). The investigators in the clinical trial for spinal cord injury are contemplating the suicide gene approach as an auxiliary method to prevent tumorigenicity (Kojima et al. 2019). All these methods will help in the careful selection of iPSC lines with the most significant level of purity that will be safe for cellular therapy.

Regarding genomic instability, a conventional method like karyotyping can detect chromosomal abnormalities, i.e., deletion, duplication, and rearrangement (Nikitina et al. 2019; Liu et al. 2020), and iPSC products with such abnormalities can be removed to ensure the safety of cells for in vivo use (Attwood and Edel 2019). However, another form of genomic instability involving minor genetic alterations, like single nucleotide variation (SNV) and copy number variations (CNV), which can be detected by the next-generation sequencing technology (like whole-genome sequencing), is challenging to analyze and may be very controversial

(Yamanaka 2020). The controversy relates to the existing difficulty in sequencing a considerable portion of our genome. Additionally, we are facing problems in accurately analyzing and interpreting the risks emanating from detected mutations as we currently lack a global consensus definition of cancer genes. The complexity of the situation gets accentuated when even healthy individuals are found to have multiple mutations in the cancer genes (Yamanaka 2020).

It is exceedingly difficult to be confident that a particular mutation detected in the iPSC products will significantly increase tumorigenicity risk following transplantation. For now, extensive tests must be carried out on the iPSC products to detect significant mutations, and only cells that pass the test should be used for cellular therapy. Additionally, following successful transplantation, the patients should be followed up and monitored for the possibility of developing a tumor. More research work is needed if we are to predict the tumorigenic potential of a detected mutation accurately.

Immunogenicity

One of the most fascinating and unique features of iPSCs is their potential for personalized regenerative cell therapy using patient-derived autologous stem cells (Omole and Fakoya 2018). The exciting prospect of generating patient-specific iPSCs that harbor a patient's genetic makeup for individualized treatment is expected to revolutionize the idea of precision medicine, an emerging concept of treatment that considers one's unique genetic background. Unlike human ESCs (hESCs) and allogeneic hiPSC transplantation, which require immunosuppression to alleviate immune rejection due to human leukocyte antigen (HLA) mismatch, autologous hiPSCs therapy will not require immunosuppression. HLAs are cell-surface molecules that help the immune system to recognize self from nonself. Zhao et al. reported that iPSC-derived teratoma elicited a T-cell-mediated immune response in syngeneic mice (Zhao et al. 2011). Their report created a controversy that autologous iPSCs seemed to be immunogenic. They attributed the immunogenicity to abnormal gene expression in iPSCs during the process of reprogramming. This controversy led to extensive research on the immunogenicity of iPSCs. De novo mutations in mitochondrial DNA (deoxyribonucleic acid) were hypothesized as the cause of immune rejections in a recently published study (Deuse et al. 2019a).

So far, other researchers have maintained that autologous iPSCs are non-immunogenic, and hence, they are well tolerated by the immune system after transplantation (Guha et al. 2013; De Almeida et al. 2014). However, if at all autologous iPSCs are immunogenic, then the underlying mechanism/s of their immunogenicity and immune rejection remain/s largely unknown. Predictably, the first iPSC clinical trial involves the transplantation of autologous iPSC-derived retinal pigment epithelial cells (iPSC-RPE) into the retina of a patient with age-related macular degeneration (AMD). The cells were transplanted without immunosuppression (Kimbrel and Lanza 2015; Mandai et al. 2017). Although there was a temporary hold of the study, as earlier mentioned, the overall perception was that the

procedure was safe and effective as the visual acuity of the patient improved, AMD progression was successfully arrested, and an area of recovery of photoreceptors was observed. More importantly, there was no evidence of tumorigenicity. Additionally, there was no evidence of immune rejection for more than 2 years after cell transplantation (Kimbrel and Lanza 2015; Mandai et al. 2017). Based on these clinical data, it was safely concluded that autologous iPSCs are well tolerated by the immune system post engraftment.

Despite the unique advantage of immune acceptance enjoyed by autologous iPSCs without immunosuppression, researchers in iPSC-based clinical trials prefer allogeneic cells due to their off-the-shelf ready-for-use availability, cost-effectiveness, and logistic considerations, unlike autologous iPSC production, which is time-intensive and more expensive. Thus, it will not be feasible to use autologous iPSCs to treat acute severe conditions, like heart failure and spinal cord injury, because time is of the essence in managing such conditions, not to mention the excessive cost of production involved therein.

Given the advantages of allogeneic cells, the researchers are addressing the immune rejection peculiar to allogeneic cell products using immunosuppressive drugs. However, this can be a very unpleasant experience for many patients, considering the many severe side effects of such drugs. Patients undergoing organ transplantation may need lifelong immunosuppressive therapy, which may be an incredibly stressful situation. Researchers are finding other ways to diminish immune rejection. One method is by doing HLA matching and using HLA-homozygous iPSC lines for cellular therapy. HLA mismatch is a major impediment in iPSC transplantation. Sugital and colleagues reported minimal immune reaction following the transplantation of major histocompatibility complex (MHC)-homozygous iPSC-RPE in MHC-matched monkeys (Sugita et al. 2016). This positive outcome was validated in a clinical trial when HLA-homozygous iPSC-RPE cells were transplanted into HLA-matched patients with macular degeneration (Sugita et al. 2020). However, similar trials conducted in the brain reveals inconsistent results, and hence, in clinical trials, this was not the case, i.e., brain (Morizane et al. 2017; Badin et al. 2019). These diverging results have been attributed to the immune-privileged status of the eye, where grafts can survive for an extended period without getting rejected. Although immunosuppressant drugs may still be required despite the HLA matching, the rate of such rejections is likely to be minimal and requires a much lesser dose and shorter duration of the immunosuppression.

With recent advances in gene-editing technology, especially CRISPR/Cas9, researchers are now able to create another method to address immune rejection by creating universal donor stem cells using the HLA cloaking method (Meissner et al. 2015; Yamanaka 2020). The universal donor stem cell will be hypoimmunogenic cell lines for off-the-shelf usage for cell-based therapy (Ye et al. 2020). MHC (major histocompatibility complex) Class I (HLA-A, HLA-B, HLA-C) molecules are inactivated by the deletion of the β2-microglobulin (β2M) gene (HLA cloaking), thus inhibiting the immune response from cytotoxic CD8+ T-cell lymphocytes (Petrus-Reurer et al. 2021). However, this approach renders the stem cells susceptible to lysis by natural killer (NK) cells. NK cell attack is prevented by knocking in

the HLA-E gene at the β2M locus (HLA-E/β2M transgene fusion) of the iPSC. This leads to the creation of universal donor stem cells that are useful for all recipients irrespective of their genetic makeup (Gornalusse et al. 2017). Deuse et al. recently devised another method of HLA cloaking. They inactivated MHC Class I and II genes by knocking out the β2M gene and CIITA (Class II, major histocompatibility complex, transactivator), respectively, and the overexpressed CD47 (proteins that prevent phagocytosis). They were able to generate a hypoimmunogenic mouse and hiPSCs that could escape immune rejection in MHC-mismatched allogeneic recipients without the use of immunosuppressants (Deuse et al. 2019b). Xu et al. proposed the C-only approach type of HLA cloaking by deleting the pair of alleles of both HLA-A and HLA-B, and one HLA-C allele, leaving behind only one HLA-C allele (Xu et al. 2019). The HLA-C-retained iPSCs evade destruction by both T cells and NK cells. The authors estimated that as few as 12 lines of such immunologically compatible HLA-C-retained iPSCs could cover 95% of the world's population. This makes the C-only approach an even more fascinating method of evading rejection since fewer cell lines are required, and it will be very cost-effective (Frederiksen et al. 2021).

Economic Issues and Biobanking iPSCs

Like most novel inventions, iPSC technology is expensive and time-intensive. Presently, it costs US $10,000–$25,000 (including the cost to expand and test for pluripotency and safety) to develop and approve a research-grade iPSC line. The time from patient recruitment to the final characterization of patient-specific iPSCs is about 6–9 months, plus a further 3–6 months to produce them on a large scale (Jacquet et al. 2013; Bravery 2015). It requires approximately US $800,000 to generate good manufacturing practice (GMP)-grade pluripotent stem cells for cellular therapy (Jacquet et al. 2013; Bravery 2015). It is generally considered that it will cost even more if recent technology, like CRISPR/Cas9, is employed as gene therapy utilizing iPSCs. Regrettably, the excessive cost of developing and validating iPSCs and the length of time involved in their generation and characterization offer a barrier that hinders their availability and use by many researchers. Cost-effective measures must be seriously considered if we are to ease the burden of the exuberant cost of iPSC generation. Hence, as discussed above, although autologous iPSC derivatives can be attractive when it comes to precision medicine, it is expensive and time-consuming. Therefore, allogeneic cell products are a good substitute as they are less expensive and could reach out to a larger number of patients. Additionally, having HLA-matched allogeneic iPSCs already generated, characterized, and tested for safety would reduce the costs and also facilitate the dissemination of the cells on time when needed.

The overall strategy should be to establish several allogeneic iPSC banks with selected lines (considering the HLA diversity of different populations) from HLA-homozygous and blood group O donors to simplify the patient-donor matching and cover most of the world populations. These iPSC lines will offer a source of

readily available transplantable cells as an off-the-shelf product for acute conditions, like spinal cord injury and heart failure. It is predicted that more than 150,000 donors are needed for screening to generate 140 HLA-homozygous iPSC lines that will be enough to cover 90% of the Japanese population (Okita et al. 2011; Umekage et al. 2019). Similarly, about 150 HLA-homozygous iPSC lines is the estimation to cover 93% of the UK population (Taylor et al. 2012). Several iPSC banking facilities are currently ongoing in Asia, Europe, and North America, storing iPSCs for research and clinical therapy purposes. The majority of the institutions involved in iPSC biobanking are nonprofit organizations that are funded by the government. This is no surprise considering the level of costs, scale, and influence associated with the iPSC technology. It is vital that such iPSC biobanks maintain high-standard regulation and high-quality and safety protocols (Ntai et al. 2017; Huang et al. 2019).

Heterogeneity

One of the issues plaguing the iPSC technology is the excessive variability within iPSCs (Cahan and Daley 2013). This variability encompasses their differentiation capability; morphology, growth, and maturation status; tumorigenicity; and genetic and epigenetic status across iPSC lines, among cells within a cell line, and amid temporal states in individual cells (Hayashi et al. 2019a). Regarding the application of iPSCs for disease modeling and regenerative cell therapy, heterogeneity remains a big concern because it can lead to poor reproducibility of research (Hayashi et al. 2019b). Genetic and epigenetic factors have been implicated as contributory factors for iPSC heterogeneity (Cahan and Daley 2013; Choi et al. 2015; Nishizawa et al. 2016). Other contributory factors for heterogeneity include donor cell type, reprogramming methods, "epigenetic memory," and culture conditions (Hayashi et al. 2019a). With gene-editing technologies, like CRISPR/Cas9, we can improve the disease phenotype of iPSCs, thus bypassing variability concerns. Additionally, researchers have tried converting a "primed" state of iPSC into a "naïve" state to tackle heterogeneity (Brons et al. 2007; Tesar et al. 2007). More research is mandatory to establish the causative factors, detection methods, reduction, and regulation of heterogeneity in iPSC lines (Volpato and Webber 2020).

Harnessing the Potential of iPSCs

The iPSC-based platform can generate stem cells for disease modeling, drug development, and regenerative cell-based therapy. At present, no iPSC-based therapy is in routine clinical use. However, we are much closer to the patient than ever. Now, there are numerous examples of iPSC applications in clinical studies and drug development. This section will summarize the recent progress in the application of iPSCs for disease modeling and drug development, and we will discuss in depth the recent ongoing iPSC-based clinical trials.

iPSC-Based Disease Modeling, Drug Discovery, and Toxicity Studies

It is vital to understand the molecular and pathological mechanisms of human diseases to develop the right therapeutic strategies to tackle them. Animal models are conventionally used to study human diseases since they offer an in vivo setting to investigate the pathological mechanisms of human diseases. Although these models have been extremely useful and instrumental over the years, however, due to interspecies differences, they have failed to recapitulate human disease phenotype fully. Hence, therapeutic strategies based on these models often fail to provide the same results when used in clinical studies. Thus, there is an urgent need for human-based disease models to complement these inefficient animal models with limited relevance to human diseases. The hiPSC-based models are ideal for the generation of patient-based cellular models and patient-specific therapeutic compounds for personalized medicine because they are readily available, accessible, and expandable in cultures; they are of patient origin and can differentiate into any disease-relevant cell type. With the advent of genome-editing technologies (e.g., CRISPR/Cas9), site-specific genetic changes in iPSCs can now be done (Shinkumaa et al. 2016). The initiation of such site-specific mutation in nondiseased iPSCs can lead to the development of isogenic iPSC lines genetically matched to patient iPSCs. These isogenic iPSC lines will recapitulate the pathology of the disease being studied and, thus, can be useful for disease modeling (Omole and Fakoya 2018).

Early-onset disorders can be modeled perfectly by iPSCs since the cells are phenotypically young (Shi et al. 2020). On the contrary, late-onset disorders are difficult to model since aging is a primary contributing factor. To model late-onset disorders, the initiation of cellular aging in the iPSCs will be necessary. To date, iPSCs have been used to model the molecular and pathological mechanisms of many diseases, for example, neurological disorders, cardiovascular diseases, cancer, etc. (Wu and Hochedlinger 2011; Devine et al. 2011; Israel et al. 2012; Cooper et al. 2012; Kondo et al. 2013; Chamberlain 2016; Yoshida and Yamanaka 2017; Kumar et al. 2018; Omole and Fakoya 2018). The iPSC models were originally single-cell type based, but these days, iPSC-based coculture models of more than one cell type are customary. For example, iPSC-based coculture of neurons and astrocytes were employed in the modeling of amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) (Di Giorgio et al. 2007; Nagai et al. 2007; Zhao et al. 2017).

Recently, there has been a paradigm shift from the standard two-dimensional (2D) monolayer adherent in vitro cell cultures to the robust "organoid" model (Eglen and Reisine 2019; Miyake and Shimada 2021). Organoids are three-dimensional (3D) multicellular in vitro tissue design that simulates their corresponding organ in vivo. In contrast to 2D models, these "mini-organ in a dish" organoid models best recapitulate the structure, organization, functions, cellular heterogeneity, architecture, and cell-cell interactions witnessed in organs in vivo, thus allowing us to model diseases perfectly (Corrò et al. 2020). In 2009, Sato et al. pioneered a study where intestinal organoids are created from adult intestinal stem cells (Sato et al. 2009). Since then, iPSCs and other stem cells have been employed to generate organoids of many organs for use in disease modeling (Spence et al. 2011; Takebe et al. 2013;

Watson et al. 2014; Tucker et al. 2014; McCracken et al. 2014; Camp et al. 2015; Takasato et al. 2015; Dye et al. 2015; Paşca et al. 2015; Mariani et al. 2015; Ogawa et al. 2015; Sampaziotis et al. 2015; Cugola et al. 2016; Otani et al. 2016; Gabriel et al. 2016; Garcez et al. 2016; Qian et al. 2016; Wiegand and Banerjee 2019).

iPSC-based models are presently being used for drug development and discovery, toxicity tests, and drug screening applications, including phenotypic screening and target-based screening (Rowe and Daley 2019; Paik et al. 2020; Pasteuning-Vuhman et al. 2021).

The phenotypic drug screening strategy involves identifying molecules that can alter the phenotype of cells in the desired manner. On the other hand, in target-based screening, we quantify the effects of compounds on a target protein during an in vitro assay (Ortuño-Costela et al. 2019). Lee et al. performed the first reported iPSC-based large-scale drug screening, wherein patient-derived iPSCs were used to model familial dysautonomia, a rare genetic disorder of the peripheral nervous system (Lee et al. 2009; Lee et al. 2012). This study proves that iPSC-based models can promote drug screening and discovery, and from then on, many iPSC-based drug screenings have been performed (Höing et al. 2012; Xu et al. 2013; Burkhardt et al. 2013; Imamura et al. 2017; Omole and Fakoya 2018; Fujimori et al. 2018; Masi et al. 2020; Shi et al. 2020). Consequently, many drug compounds for the treatment of several diseases have been analyzed, and a few candidate drugs thus identified have proceeded to clinical trials for safety and efficacy testing (Naryshkin et al. 2014; Mullard 2015; Bright et al. 2015; Hino et al. 2015; Hosoya et al. 2017; Morimoto et al. 2019; Imamura et al. 2019; Masi et al. 2020; Shi et al. 2020). For example, Imamura et al. conducted a phenotypic drug screening with existing Src/c-Abl inhibitors using hiPSC-derived motor neurons from ALS patients (Imamura et al. 2017). This study led to the discovery of bosutinib and, thus, drug repurposing since the drug was previously approved by the Food and Drug Administration (FDA) to treat chronic myelogenous leukemia. Now, a trial to evaluate the safety, efficacy, and tolerability of bosutinib in ALS patients is underway (Trial registration number: UMIN000036295) (Imamura et al. 2019). Similarly, Hideyuki Okano's group has successfully identified ropinirole hydrochloride as a candidate drug to treat ALS following a drug screening test using patient-derived iPSC spinal motor neurons (Fujimori et al. 2018). In December 2018, they commenced a phase I/IIa clinical trial (ROPALS trial) to explore the safety, efficacy, and tolerability of the drug (Trial registration number: UMIN000034954) (Morimoto et al. 2019).

A recent trend in iPSC-based modeling and drug discovery is the organ-on-a-chip (OOC) technology (Wu et al. 2020; Paloschi et al. 2021). OOC is a 3D multicellular microfluidic device consisting of a mixture of iPSCs and engineered extracellular matrix biomaterials. OOC helps to recapitulate the physiological characteristics of human tissues/organs in vivo, allowing us to measure physiological parameters for drug effects and toxicity assessment. Compared to experimental animal models, OOC is a cheap and less time-consuming alternative to the contemporary methods for drug testing during the process of drug development. Many OOCs of different organs have been developed to serve as a platform for drug testing (Jodat et al. 2018).

iPSC-based models have been employed for the cytotoxicity screenings of therapeutic drugs (Deshmukh et al. 2012). High on the list of such drug toxic effects is cardiac and hepatic toxicities (Csöbönyeiová et al. 2016). Itzhaki and team used iPSC-cardiomyocytes from long QT syndrome patients to assess the efficacy and cardiotoxic effects of existing and new drugs (Itzhaki et al. 2011; Omole and Fakoya 2018). The use of patient-specific iPSCs for cardiac disease modeling has been excellently described by Cagavi et al. (2018).

Finally, the immense potential of iPSCs in disease modeling, drug discovery, and cytotoxicity testing portends a fruitful and promising near future in the pharmaceutical industry and research field.

iPSC-Based Cell Therapy – Ongoing Clinical Trials

The iPSC platform offers an exciting opportunity to generate functional, healthy cells to replace damaged or injured tissues, promote endogenous regenerative repair, and restore the functionality of an organ. Cellular therapy via iPSC transplantation is a novel technique that is no longer futuristic as the dream is now a reality (Buccini et al. 2012; Ahmed et al. 2011b). Since the generation of mouse and human iPSCs in 2006 and 2007, respectively, earnest efforts have gone into iPSC cell therapy applications via several preclinical studies to test their therapeutic response. Animal models have been invaluable in helping us answer the vital question: will the grafted cells integrate into the host and fulfill their function correctly? A proof of concept was provided by two early groundbreaking preclinical studies on the road to the realization of this dream. In 2007, Jaenisch et al. used a gene-targeting approach to repair a disease-causing mutation in the mouse iPSCs' humanized model of sickle cell anemia (SCA) (Hanna et al. 2007). The transplantation of the repaired SCA-iPSCs successfully corrected the disease phenotype. In 2008, Wernig et al. (from the Jaenisch research group) transplanted iPSC-derived dopaminergic neurons into a Parkinson's disease (PD) rat model and reported functional integration of the graft with improvement in behavioral symptoms (Wernig et al. 2008). These two landmark preclinical studies motivated the stem cell research community into further exploring iPSC therapy in humans.

The number of interventional clinical trials (involving the transplantation of iPSCs into humans) can show how far stem cell scientists have gone in the long road of translating the iPSC technology from the bench to the bedside, giving us a significant hope for the routine clinical use of iPSCs in the future via cellular transplantation as a novel therapeutic option.

In their analysis of a systematic multidatabase search performed in August 2019, Deinsberger et al. found 131 clinical trials involving human pluripotent stem cells; 77.1% (101) of these clinical trials were observational, while 22.9% (30) were interventional; 73.3% (22) of the interventional studies were hESCs based, while hiPSCs were used in only 26.7% (**8**) (Deinsberger et al. 2020). Their systematic search was based on databases "ClinicalTrials.gov" and "International Clinical Trials Registry Platform" (ICTRP) from the World Health Organization (WHO). Similarly,

a recent systematic search conducted by Yamanaka in September 2020 using databases "ClinicalTrials.gov" and UMIN Clinical Trials Registry reveals 15 interventional clinical trials based on hiPSC transplantation (Yamanaka 2020). We combined the data from these two studies and produced **17** past and presently ongoing interventional clinical trials involving hiPSCs (Fig. 1). Additionally, the two studies reveal more than a 100% increase in the number of hiPSC-based interventional clinical trials during the last year, suggesting that hiPSC-based clinical trials are rapidly growing as we journey into the second decade of their discovery. We will now do a review of a few of these remarkable hiPSC-based interventional clinical trials, using it to confirm their potential for regenerative medicine.

So far, more noticeable progress has been made for age-related macular degeneration (AMD). In 2009, Carr et al. demonstrated, for the first time, visual function recovery in a rat model's retina following the transplantation of iPSC-RP (Carr et al. 2009). Spurred on by this preclinical study, in 2014, Takahashi et al. at Riken Institute in Japan set their aim on clinical applications by developing autologous iPSC-derived RPE (retinal pigment epithelial) cell sheets that were optimized to meet clinical-grade requirements for the treatment of AMD (Kamao et al. 2014). Upon the transplantation of these cell sheets into an experimental primate model,

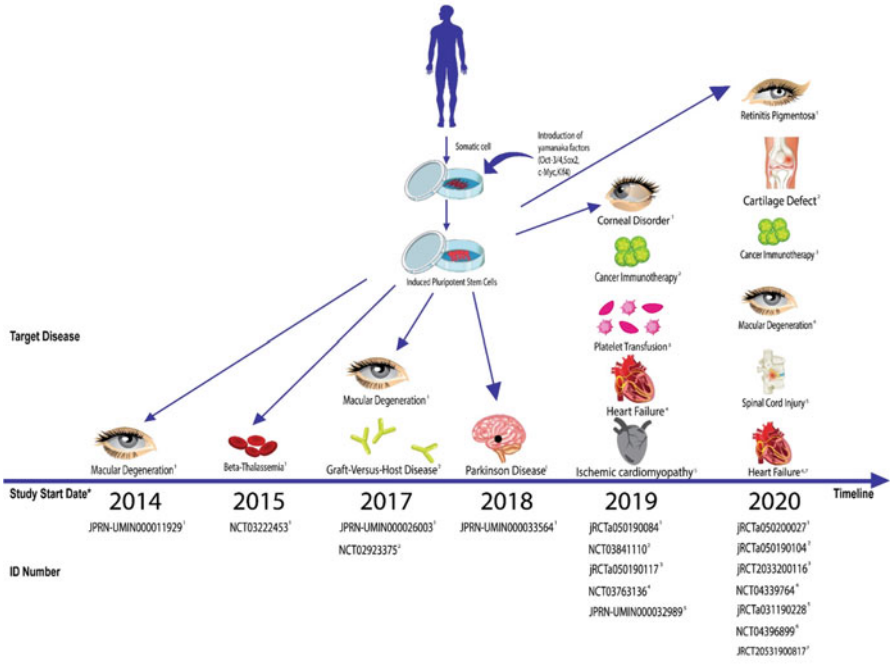

Fig. 1 Interventional clinical trials involving human-induced pluripotent stem cell (hiPSC)-based therapies. (*Study start date is defined as the actual date on which the first participant was enrolled in a clinical study)

there was no immune rejection of the transplanted cells or tumor formation. This successful large experimental animal preclinical study led to the first hiPSC clinical trial in the same year. Takahashi et al. grafted an autologous iPSC-RPE cell sheet into the retina of a 77-year-old woman with AMD, without immunosuppression (Garber 2015; Kimbrel and Lanza 2015; Scudellari 2016; Mandai et al. 2017; Attwood and Edel 2019). Though the trial was halted due to mutation noted in the iPSCs of the second patient, the overall conclusion after 2 years posttransplantation is that the trial was successful (Kimbrel and Lanza 2015; Apatoff et al. 2018). Takahashi et al. commenced another clinical research in 2017 using HLA-matched allogeneic iPSCs-RPE cells in a 60-year-old man with AMD (Cyranoski 2017).

Additionally, an iPSC bank was established at the Center for iPS Cell Research and Application (CiRA) in Japan (Umekage et al. 2019). There are three other most recent ongoing iPSC-related clinical trials for eye disorders. Firstly, in July 2019, Nishida et al. at Osaka University, Japan, initiated the first-in-human clinical research of iPSC-derived corneal epithelial cell sheet transplantation for patients with limbal stem-cell deficiency, a type of corneal disorder. One month post-transplantation, the patient's vision improved (Cyranoski 2019). Secondly, in June 2020, Yasuhiko et al. at Kobe City Eye Hospital transplanted allogeneic iPSC retinal sheets for patients with retinitis pigmentosa. Finally, in September 2020, Wiley et al. of the National Eye Institute (NEI), National Institutes of Health (NIH), USA, commenced a phase I/IIa trial for the autologous transplantation of iPSC-RPE cells for geographic atrophy associated with AMD (Fig. 1).

Besides the treatment of eye disorders, much attention has also been directed toward applying iPSC-based products to manage neurological disorders, with Parkinson's disease (PD) being the most advanced (Doi et al. 2020). Takahashi's research group at Kyoto University reported in 2017 that human iPSC-dopaminergic progenitor cells survived and functioned as midbrain dopaminergic neurons in a primate model of PD (Takahashi 2017; Kikuchi et al. 2017). The following year, the same group commenced the first clinical trial to treat PD using hiPSC-derived dopaminergic neurons (Cyranoski 2018a; Stoddard-Bennett and Reijo Pera 2019). Whether PD will be the first neurodegenerative disease to be cured by iPSCs remains an interesting question.

On the oncological front, iPSC-derived cells are also helpful for patients with cancers. In February 2019, Fate Therapeutics, an American Company, commenced a clinical trial involving the allogeneic iPSC-derived NK-cell-based therapy for up to 64 patients with advanced solid tumors (Nianias and Themeli 2019). Additionally, the Australian company Cynata Therapeutics launched a clinical trial in March 2017 involving the infusion of allogeneic iPSC-derived mesenchymal stem cells for the treatment of steroid-resistant graft-versus-host disease (GVHD). Phase I of the trial was completed in August 2018 with positive results, and research is underway to commence the phase 2 trial (Rasko et al. 2019).

iPSC has been applied as a prospective therapy for other diseases. For heart disorders, Sawa et al. at Osaka University got the approval for a clinical trial to transplant allogeneic iPSC-derived cardiomyocyte sheets in three patients with

ischemic cardiomyopathy (Cyranoski 2018b). There are three other ongoing clinical trials on heart-related disorders (Fig. 1; ID-Numbers-NCT03763136, NCT04396899, and JRCT20531900817).

Similarly, other applications include spinal cord injury (jRCTa031190228), cartilage defect (jRCTa050190104), and thrombocytopenia disease (jRCTa050190117).

Conclusion

The iPSCs' platform has become an excellent and powerful tool for understanding and treating human diseases. During the last decade and a half, considerable progress has been made to generate organ-specific cells and tissues from iPSCs, several of which have been moved into clinical trials. We are confident that many advancements and excitements await us during the second decade of iPSC technology as we take a further leap from bench to bedside. Though some barriers still exist that block the full realization of the potential of iPSCs, the authors strongly believe that many of these are technical, and with time, "this too shall pass away." We are confident that the iPSC platform will become a viable treatment option for many diseases, besides iPSCs' diagnostic applications and use in drug development in the not-so-distant future.

Cross-References

- ▶ Common Ethical Considerations of Human-Induced Pluripotent Stem Cell Research
- ▶ Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs) as a Platform for Modeling Arrhythmias

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Human Stem Cell Differentiation In Vivo in Large Animals

31

John S. Pixley

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Abstract

The discovery of common placental circulation between twins coupled with the development of erythrocyte profiling in cattle allowed Ray Owen (Science 1945) to determine that dizygotic twins were chimeric with their sibling's blood cells after birth. Thus, the author concluded that self-tolerance is acquired during fetal development through the intermingling of sibling cells via the placental circulation and, as a corollary, immune tolerance to self is not genetically determined or innate. Pixley's group has also exploited this process to engraft human stem cells in a large animal (sheep) via in utero transplantation (IUT). Advantages to large animal investigation include long life span, large size, and serial sampling. Using parallel studies in developing sheep fetuses, stem cell engraftment receptivity and sheep lymphoid ontogeny were assayed longitudinally. They were able to identify an engraftment window and propose their antigen exposure model to explain

J. S. Pixley (✉)
Texas Tech University Health Science Center, Lubbock, TX, USA
e-mail: john.pixley@ttuhsc.edu

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acquisition of immune tolerance to self as chimeric animals display lifelong immune tolerance to the graft. The subsequent chimeras yield human hematopoietic, islet, hepatic, cardiac, and gastrointestinal cellular elements in situ. Circulating human proteins (IgM, albumin, factor VIII, C-peptide, and alpha-fetoprotein) are detected years after transplantation. Therefore, a fully tolerant large animal host provides an ideal method to test human stem cell differentiation in vivo. This chapter will discuss evidence for immune tolerance and potential advantages of IUT in assaying stem cell differentiation in vivo (in comparison to immunodeficient animal models). Limitations to stem cell differentiation following IUT will be discussed as well.

Keywords

Fetal tolerance · In utero transplantation · Self/non-self discrimination · Pluripotency · Stem cell

Abbreviations

CAR	CXCL-12-abundant reticular cells
CD	Cluster of differentiation or cluster of designation or classification determinant
CFU-F	Colony-forming unit fibroblast
CXCL	CXC chemokine ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FISH	Flourescent in situ hybridization
G_0	Gap 0
G6PD	Glucose-6-phosphate dehydrogenase
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
HSC	Hematopoietic stem cell
IUHST	In utero stem cell transplantation
IUT	Intrauterine transplantation
LCA	Lymphocyte common antigen
MSC	Mesenchymal stem cell
PCR	Polymerase chain reaction
SC	Stem cell
Tx	Transplanted

Introduction

The Nobel Prize in Physiology or Medicine was awarded in 1960 jointly to Sir Frank Macfarlane Burnet and Peter Brian Medawar "for the discovery of acquired immunological tolerance." Their experiments and theories were prompted by Ray Owen's observations in cattle (published in 1945) that dizygotic twins were chimeric with their sibling's blood cells after birth as a consequence of intermingling of sibling

cells via the placental circulation. Owen's report comprising two written pages forms the modern understanding of immune tolerance, the existence of the hematopoietic stem cell (HSC) (in concert with H.E. Jordan), and stem cell engraftment. Peter Medawar thought Ray Owen should have received the Nobel Prize (Crow 1996; Owen 1945; Jordan 1942). The main idea conveyed was that self-tolerance is acquired during development and not genetically determined.

Long-Term Transplantation Tolerance Following Intrauterine Transplantation (IUT)

Subsequent research using bone marrow cell populations in culture or following transplantation in small and large animals has advanced the general understanding of SC biology. Numerous scientific and lay media publications have spawned considerable excitement concerning SCs' potential to reverse numerous disease processes and/or repair diseased organs. While preliminary studies involved in vitro assays of SC differentiation, transplant studies to assess SC behavior in vivo have proven crucial to advancing the field. To obtain engraftment and expression of allogeneic or xenogeneic SCs in live-born animals, methods are required that generate immune tolerance to the graft in the host or recipient; as failure to induce tolerance will result in graft failure. Methods used to achieve this include irradiation, pharmacologic agents (such as alkylating agents), or using immunodeficient mice as host. Alternatively, in utero SC transplantation (IUSCT) based on Owen, Medawar, and Billingham's observations allows natural events in immune development to achieve graft tolerance. Humanized mice are, in essence, the extension of the immunodeficient transplant model for the in vivo study of human cell behavior and have recently been reviewed (Sutherland et al. 1989; Spangrude et al. 1988; Iscove and Nawa 1997; Ullao-Montaya et al. 2005; Bhatia et al. 1997; Flake et al. 1986; Zanjani et al. 1992; Fujiwara 2018; Stripecke et al. 2020).

Early on to assess proof of principle, IUSCT was attempted in mice and sheep with the long-term goal of attempting to correct congenital disease by transplanting normal cells. The procedure involved preparing donor SCs and transplanting them into the fetal abdomen, although venous and intra-cardiac transplantation has been performed more recently. [Early experiments used unpurified SC populations (i.e., fetal liver).] Gestational timing of the procedure is crucial to the achievement of donor cell engraftment and subsequent expression by inducing tolerance to the graft in parallel with fetal development of tolerance to self.

Flake and Zanjani confirmed that self/non-self discrimination occurs during development by demonstrating lifelong engraftment/expression of both allogeneic and xenogeneic (human) SCs in sheep following IUT. By using a large experimental animal (sheep), Flake and Zanjani were able to vary transplantation temporally during gestation, and it became clear that the tolerance formation took place at a distinct time in development (originally noted by Silverstein also in sheep) (Flake et al. 1986; Zanjani et al. 1992; Silverstein et al. 1964). This period was originally termed the "window of opportunity" based on the hope that the procedure could be

applied clinically to treat childhood hematolymphoid diseases. Recently, Pixley and colleagues identified the fetal maturational events of thymic vascularization and demarcation as clarifying when in development ontogenic programming of self-tolerance occurs. The study of ovine fetal development demonstrated that lymphocyte lineage markers were present in peripheral organs early in gestation but differentiated CD45 co-expression (a marker for cell maturity) was only noted in the thymus. Parallel IUT experiments using allogeneic and human SCs confirmed long-term engraftment following transplantation occurs coincident with the above-noted maturational events in the thymus. Indeed, deletion was readily detected in most lineages. Engraftment kinetics was similar using either allogeneic or xenogeneic donor cells, and these developmental events were termed "the engraftment window" (Skopal et al. 2009; Pixley and Zanjani 2013; Toivanen et al. 1981) (Figs. 1 and 2a, b).

IUSCT has also been successfully performed using allogeneic and xenogeneic SC in mice, with the timing of successful transplantation also linked to vascularization and demarcation events in the mouse fetal thymus. Using either large or small animals, tolerance has been demonstrated (Pixley and Zanjani 2013; Toivanen et al. 1981; Carrier et al. 1995; Peranteau et al. 2002; Pallavicini et al. 1992; Pixley et al. 1998; Kim et al. 1999; Laje et al. 2006; Fairchild and Waldmann 2000).

While various research groups have confirmed that thymic deletion is operative during the engraftment window, cellular (or peripheral) tolerance in the lymphocyte lineage and natural killer cell lineage is likely established as well (Table 1). Indeed, a variety of experimental manipulations have shown the ability to improve graft expression after birth based on evidence that the host is fully tolerant to the graft (Pixley and Zanjani 2013; Peranteau et al. 2002; Almeida-Porada et al. 2007). In addition, Mintz and Blazar confirmed the hypothesis that IUT could cure newborn hematolymphoid disease following transplantation of normal allogeneic bone marrow cells into diseased mice (Fleischman and Mintz 1979; Blazar et al. 1995a, b).

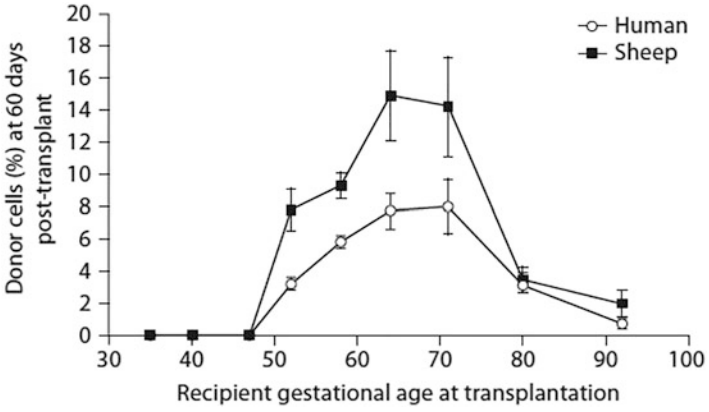

Fig. 1 Engraftment receptivity is gestational age-dependent. For both allo- and xenotransplantation, cells were transplanted at gestational ages 35, 40, 47, 52, 58, 64, 71, 80, and 92. Independent of donor source, there is an absence of engraftment if IUHSCT is performed prior to day 52; receptivity peaks between days 64 and 71 of gestation and then rapidly falls. SEM is shown as error bars. (Skopal et al. 2009, with permission)

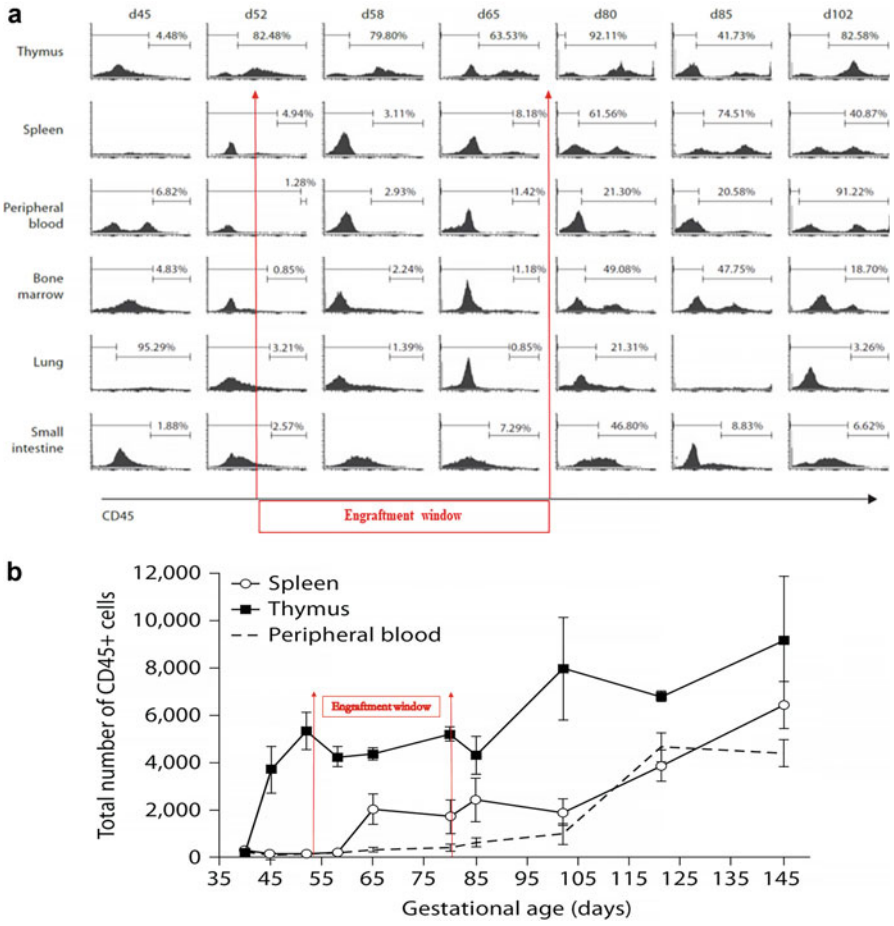

Fig. 2 (**a**, **b**) Expression of CD45 (LCA) on cells of the thymus and peripheral organs during gestation. Expression is found early in the thymus (beginning at day 52), followed by expression in the spleen and PB after the engraftment window closes (day 80). (**a**) Representative histograms showing percent expression of CD45 on the fetal ovine thymus, PB, spleen, bone marrow, lung, and small intestine at selected time points throughout gestation. (**b**) Cumulative data demonstrating expression of CD45 on cells from the fetal ovine thymus, spleen, and PB throughout gestation. SEM is shown as error bars. (Skopal et al. 2009, with permission)

What Is a Stem Cell?

As originally proposed by Till, McCulloch, and Siminovitch, a SC possesses three distinct properties: the ability to self-renew, undergo multi-lineage differentiation, and exhibit extensive proliferative capacity (Till et al. 1964). This theorem remains operational today. Thus, premature differentiating cells which lose the ability to self-renew are termed progenitors. While this study assessed hematopoietic reconstitution, it is now

Table 1 Groups of six sheep were assessed for tolerance to allogeneic hematopoietic stem cells (HSCs) and then re-transplanted after birth with the same donor HSCs

Allogeneic sheep HSC render recipient sheep[a] tolerance following in utero transplantation		
Stimulator	Responder	Stimulation index[b]
Donor	Donor	0
Recipient	Recipient	0
Donor	Recipient	0–8
Recipient	Donor	58 ± 11
Pooled	Donor	69 ± 12
Pooled	Recipient	78 ± 12
Postnatal infusion of allogeneic same donor HSC augments engraftment in tolerant sheep[c]		
% Donor cells at birth	*n*	% Increase[d]
6–10	4	86 + 29
11–15	5	63 + 22
>15	4	21 + 11

Pixley and Zanjani (2013), with permission
HSCs hematopoietic stem cells
[a]Representative sample of six chimeric lambs
[b]Variation of mixed lymphocyte reaction (MLR) previously reported
[c]Tolerance determined via MLR
[d]Assessed 6 months after postnatal stem cell infusion (3 × 108 cells/kg) in 13 chimeric lambs rendered tolerant

recognized that there exists a large variety of tissue-based SCs. While all SCs must be capable of self-renewal, the differentiating capacity is thought to vary from totipotent (a characteristic of embryonic SCs) to pluripotent to multipotent as the organism develops (where multipotent represents "somatic" or adult SCs). The differentiation capacity of somatic SCs is quite varied as will be seen later but not totipotent (Fig. 3a, b).

The marrow SCs had been theorized for almost half a century prior to Till and McCulloch's observation in 1964, and it was known that murine hematopoiesis could be reconstituted following lethal radiation with whole bone marrow. Interestingly, the first successful bone marrow transplantation was performed in 1959 in two patients using whole bone marrow as donor from identical twins (to avoid graft rejection and or graft-versus-host disease) into recipients irradiated for leukemia prior to the report by Till and McCulloch (Pixley 2020).

Since then, it is now recognized that there is a varied SC pool that exists in organs other than marrow. Research has focused most specifically on mesenchymal stem cells (MSCs), which possess diverse differentiation features but retain the capacity to self-renew. For example, MSCs are present in the bone marrow where they provide a support function for hematopoietic SCs, but also possess hematopoietic regeneration capability, may be found in remote tissues, and can differentiate extensively in vitro and in vivo. Furthermore, they are easily manipulated; can be expanded in culture as opposed to the hematopoietic SC, which tends to lose self-renewal capacity in culture; and are free from the ethical concerns with the use of human embryonic SC (Ratajczak 2018; Tavassoli and Crosby 1968; Friedenstein et al. 1974; Pittenger et al. 1999; Kincade 2010; Moore 2012).

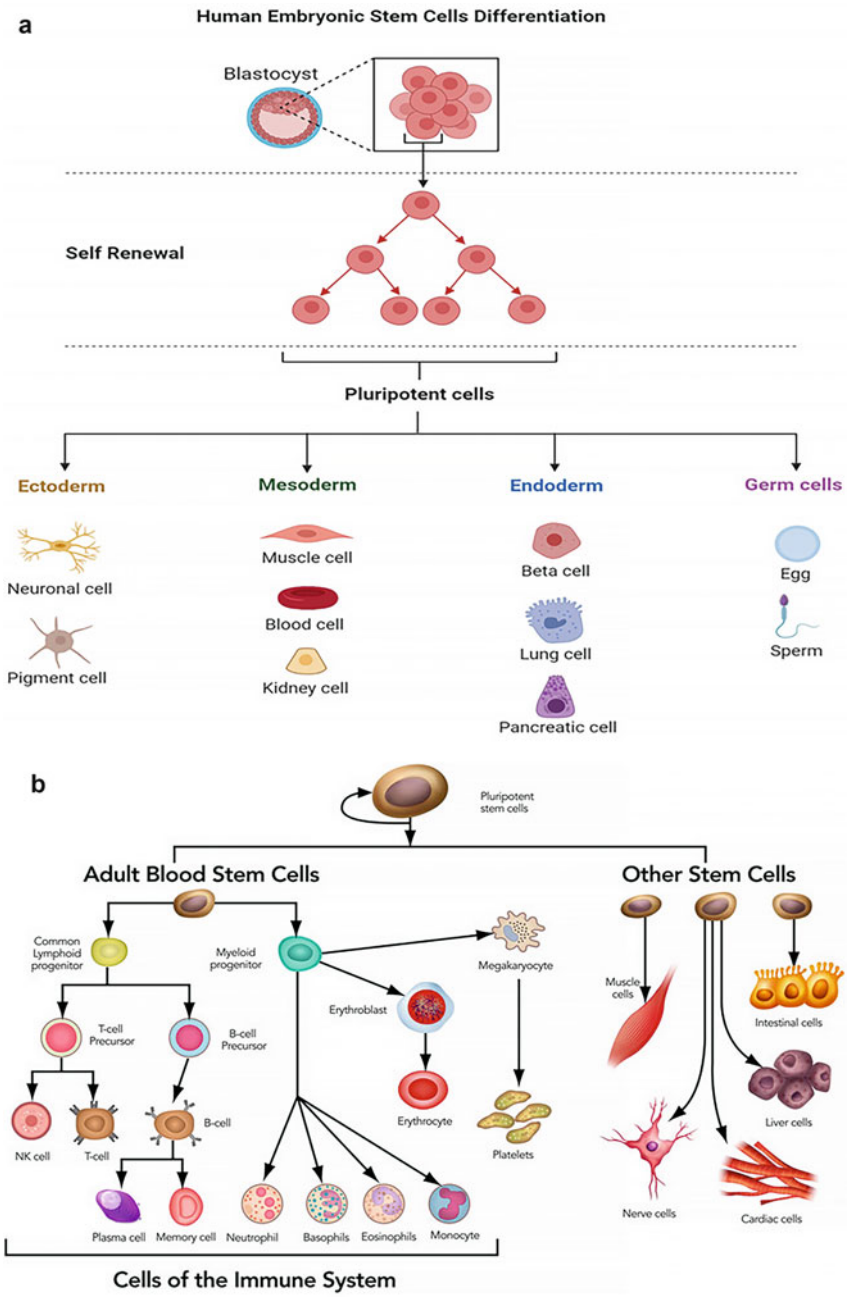

Fig. 3 (**a**) Representation of embryonic stem cell differentiation. (Reprinted with permission from Microbe Notes. "Stem Cells- Definition, Properties, Types, Uses, Challenges" October 12, 2020 by Anupama Sapkota). (**b**) Representation of somatic stem cell differentiation. (Reprinted by permission from the Maharaj Institute of Immune Regenerative Medicine website)

Why a Large Animal?

Simply put, large animal experimentation offers superior size, long-term serial monitoring, and investigations likely impossible in small animals with short life spans. It is recognized that mice and/or rats including inbred strains, gene knockout, transgenic, and disease induction have provided innumerable useful observations on mammalian biology and will continue to be the core experimental animal system. Yet, as noted above, the original observation regarding the existence of hematochimeras was made in cattle.

Another example of how large animal experimentation can expand the knowledge of stem cell behavior was performed by Abkowitz. Here, autologous transplantation was performed, and hematopoietic recovery was observed over 4.5 years. Taking advantage of genetic mosaicism of the X-linked G6PD enzyme in female cats, the relative contribution to hematopoiesis by each of the stem cell mosaics was observed. While considerable variation in relative contribution to hematopoiesis by SCs containing one or the other mosaic was noted early (<1.5 years after transplantation), later examination revealed considerably less variation such that one cat expressed cells of only one mosaic. As the experimental design called for transplantation of a limited number SCs, the authors concluded the early disequilibrium was due to extensive SC renewal necessary to replenish the SC pool. When equilibrium was reached, a small number of self-renewing SCs were capable of extensive hematopoiesis (Abkowitz et al. 1995).

It has been argued that large animals offer a biologic system more akin to humans, and observations may be more translatable to human physiology. There has been considerable interest in developing IUT as a therapeutic tool to treat childhood diseases. This will require refinement of the technique in larger animals. While successful engraftment has been demonstrated in primates and many large animal species, the clinical application requires further modification and understanding, which will be discussed below. At the minimum, a large experimental animal model offers confirmatory evidence regarding observations in small animals.

IUT as an Experimental Tool to Study SC Biology or What Is a Stem Cell Revisited

The Hematopoietic SC (HSCs)

The standard stem cell transplantation assay is performed in irradiated normal or immunodeficient mice (with or without radiation) to render the animal tolerant/receptive to the graft. The experimental animals are assayed for peripheral blood multi-lineage donor cell expression using a variety of methods to differentiate donor cells from the recipient in the allogeneic system. Xenogeneic mouse transplantation systems require that the animal be immunodeficient to enable engraftment. Much has been learned from these assay systems (Spangrude et al. 1988; Iscove and Nawa 1997; Ullao-Montaya et al. 2005; Bhatia et al. 1997).

Due to durable tolerance to the graft, IUT performed in sheep has enabled in vivo analysis of putative stem cell populations. While surface expression of human CD34 is used as a SC phenotype (in clinical transplantation), it is also known that SC potential is present in a minority of CD34+ cells. This was explored using two different CD34+ populations using IUT in sheep. The first population was CD34+/lineage−/CD38−; the second was CD34+/lineage−/CD38+. Evidence for long-term persistence and serial transplantation (re-transplantation of donor cells into a second recipient) were used to assay human cell activity in sheep recipients (Civin et al. 1996). Serial transplantation is used to differentiate short-term (progenitors) from long-term repopulating cells (presumably SCs) popularized by transplantation experiments in irradiated mice, as discussed earlier (Iscove and Nawa 1997). Thus, long-term repopulating cells are thought to represent true hematopoietic SCs capable of self-renewal. As can be seen in Fig. 4, long-term persistence and presence of re-transplantable human hematopoiesis were observed only in animals transplanted with CD34+CD38− cells. These studies were extended and demonstrated that the hematopoietic SC population included CD34− cells and that the expression of CD34 on putative stem cell populations was reversible, a finding observed in mice as well (Zanjani et al. 1998, 2003). An exhaustion strategy was used to differentiate persistence of the graft using either CD34+ or CD34-lineage-negative cells using late-acting hematopoietic growth factors. Early exhaustion (loss of long-term repopulating cells) was seen in the CD34+ population (Zanjani 2000). As CD34 is used as the marker for clinical bone marrow transplantation, it is interesting to note that no late graft failures have been reported despite the above observation. Thus, IUT in large animals is a powerful method to assay human stem cell behavior in vivo.

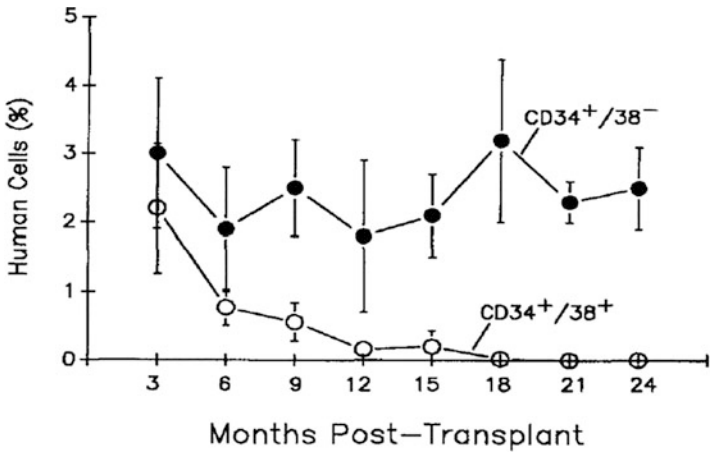

Fig. 4 Long-term persistence of human hematopoiesis in human/sheep chimeras transplanted with adult human marrow CD34+/CD38− cells, but not CD34+/CD38+ cells. Human engraftment was determined by karyotype analysis. Means (+1 SEM) of results obtained from three CD34+/CD38− or five animals engrafted with human cells are plotted. (Civin et al. 1996, with permission)

As noted in Fig. 3b and discussed earlier, it was thought that, for example, hematopoietic SCs were somatic stem cells and did not possess the ability to cross developmental germline boundaries (plasticity). However, specific SC transplant and in vitro studies suggested that indeed this was not the case. For example, HSCs were reported to generate hepatocytic and pancreatic islet activity (and other organ phenotypes) in mice following transplantation (Almeida-Porada et al. 2010; Ianus et al. 2003; Iskovich et al. 2012; Parekh et al. 2009; Petersen et al. 1999; Theise et al. 2000).

Much controversy accompanied these observations. The thrust of critiques denying SC plasticity suggested: (1) the observations were not reproducible and (2) evidence of plasticity was due to fusion of donor SCs with mature or differentiated cells of the respective organ, for example, liver cells or endocrine pancreas. In general, divergence in the methods used explains some of the observed discrepancies (Almeida-Porada et al. 2010; Ianus et al. 2003; Iskovich et al. 2012; Parekh et al. 2009; Petersen et al. 1999; Theise et al. 2000; Wagers et al. 2002; Vassilopoulos et al. 2003; Wang et al. 2003; Choi et al. 2003; Theise 2010; Lechner et al. 2004; Taneera et al. 2006). SC differentiation into alternate lineages was thought to be promoted by tissue injury where the injured recipient cells would provide paracrine support for donor SC differentiation. To address this issue, Sharkis and colleagues demonstrated hepatocytic activity of highly purified marrow HSC using a trans-well membrane to preclude fusion in an injury model for SC plasticity (Fig. 5) (Jang et al. 2004). The advantage of finding human hepatocytic and pancreatic activity following human SC transplantation in utero is that there is no injury stimulus involved therein. Using fluorescent in situ hybridization (FISH) probes to differentiate donor from recipient DNA in hepatic cells, no evidence or merging of the respective dyes was seen in representative samples. In this study, human blood vessels and biliary cells were seen supporting full-fledged differentiation of the transplanted SC (Fig. 6a, b, Almeida-Porada et al. 2004).

The Mesenchymal Stem Cell (MSC)

As mentioned earlier and shown in Fig. 3b, a variety of self-renewing/proliferating cells have been identified in multiple organs. Of these, considerable interest has been generated by the identification of MSCs first identified in marrow by the use of explant studies that were able to separate the marrow niche or support structures from hemopoietic elements, which entered the graft after the supporting structures matured. A stromal-derived SC was identified using in vitro colony assays. Termed colony-forming unit-fibroblast (CFU-F), these bone marrow stroma-derived cells demonstrated clonal proliferation, were found to be extremely adhesive and slowly proliferative, and retained differentiation capacity on successive passaging. Now termed mesenchymal stem cells (MSCs), they are identified by their ability to form osteoblasts, adipocytes, and chondroblasts in vitro following isolation by plastic adherence (CFU-F) (Table 2) (Tavassoli and Crosby 1968; Friedenstein et al. 1974; Pittenger et al. 1999; Kincade 2010; Moore 2012). Bone marrow-derived MSCs, as

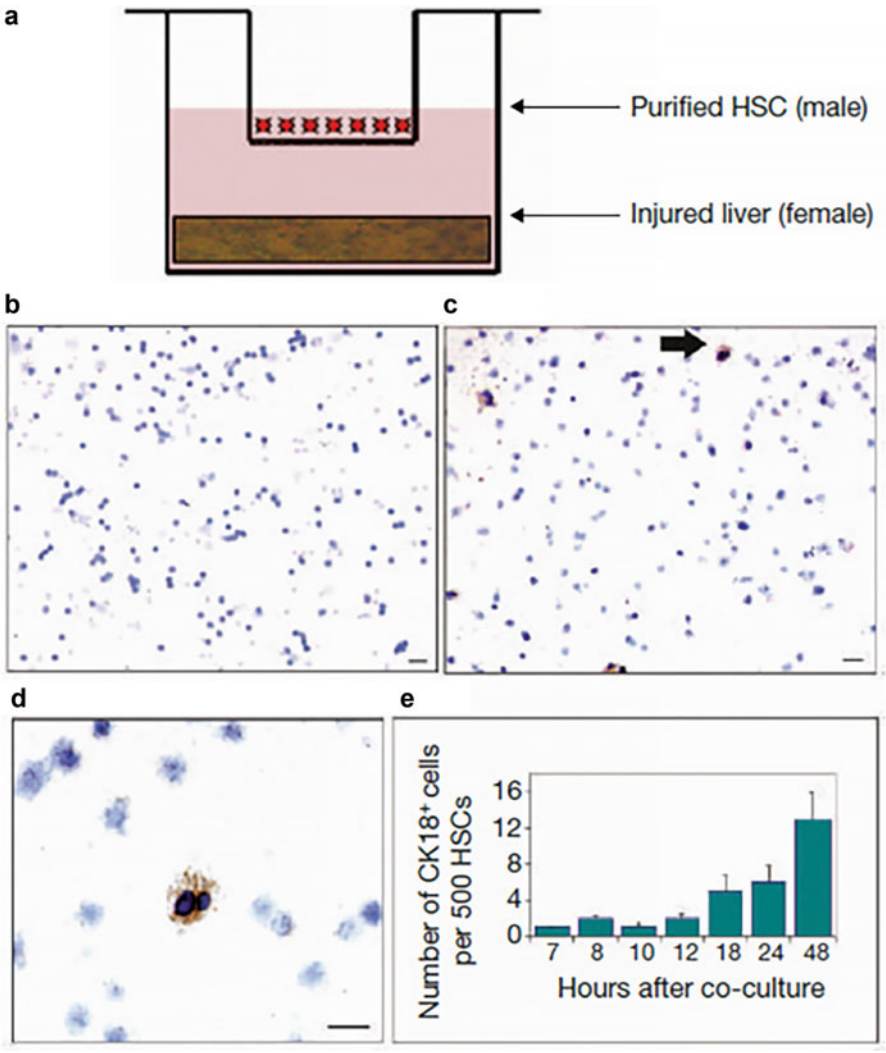

Fig. 5 Differentiation of HSCs. (**a**) Experimental design of co-culture. HSCs (Fr25lin-PKH+ homed to marrow cells) were placed in the upper chamber and incubated with injured minced liver tissue in the lower chamber for 7–48 h. (**b**–**d**) Immunocytochemical staining for hepatocyte-specific marker CK18. Scale bars, 10 um. (**b**) HSCs cultured alone did not stain positively with antibody against CK18. (**c**) HSCs co-cultured with CCL_4-treated liver for 48 h (original magnification, ×10). (**d**) The CK18-positive, bi-nucleated cell indicated by an arrow is seen at higher power (original magnification, ×40). (**e**) Frequency of CK18 staining over time in culture. (Jang et al. 2004, with permission)

opposed to other tissue-derived MSCs, are the most well-characterized and best understood subpopulation. Their crucial role in marrow function is described in Fig. 7.

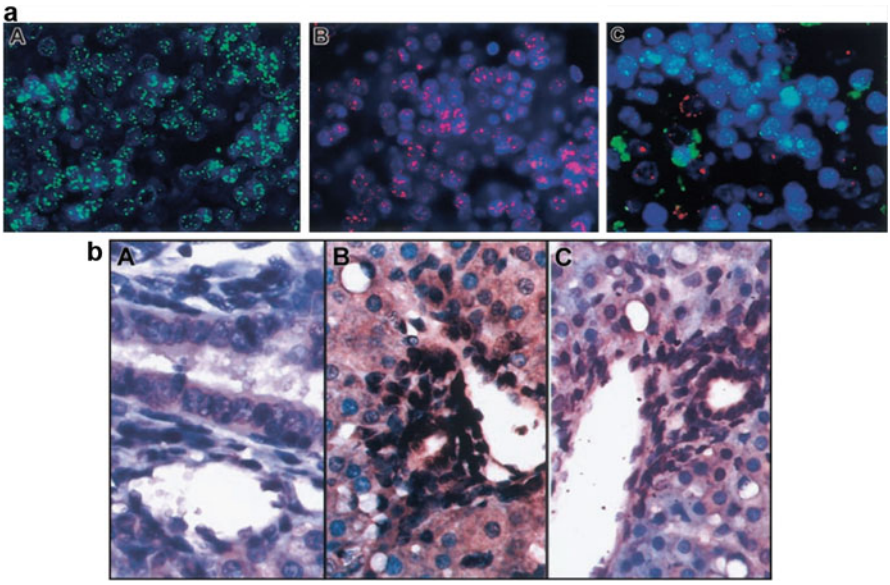

Fig. 6 (**a**) Human hepatocyte generation in the fetal sheep model is not caused by cell fusion. In situ hybridization of liver sections of (A) a control sheep hybridized with human pericentromeric repeat (red) and sheep pericentromeric repeat (green) probes and (B) control human liver also hybridized with both probes. As can be seen, the probes were completely species specific. Control sheep displayed only the green signal from the sheep probe, whereas the control human liver exhibited only red fluorescence from the human probe. (C) In situ hybridization of a representative liver section of a chimeric sheep. Human hepatocytes within the sections exhibited pure red fluorescence, demonstrating that they contain only human DNA and not sheep DNA, whereas the sheep hepatocytes exhibit only green fluorescence. (Almeida-Porada et al. 2004, with permission). (**b**) In situ hybridization shows that cells within the vessel walls and biliary ducts are of human origin. Combined in situ hybridization and immunohistochemistry were performed on liver sections from sheep that underwent transplantation using a probe specific for the human Alu sequence and an antibody specific for human CD45. (A) Control (no transplantation) sheep. (B, C) Two different sections show numerous cell nuclei within the vessel walls and biliary ducts stained dark brown, demonstrating that they are of human origin. None of these cells exhibit red staining for CD45. (Almeida-Porada et al. 2004, with permission)

Table 2 International Society for Cellular Therapy minimal criteria for human MSC

1. Adherence to plastic in standard culture conditions	
2. Phenotype	
Positive (>95%)	Negative (<2%)
CD105	CD45
CD73	CD34
CD90	CD14 or CD11b
	CD79α or CD19
	HLA-DR
3. In vitro differentiation: osteoblasts, adipocytes, and chondroblasts (demonstrated by staining of in vitro cell culture)	

Dominici et al. (2006), with permission

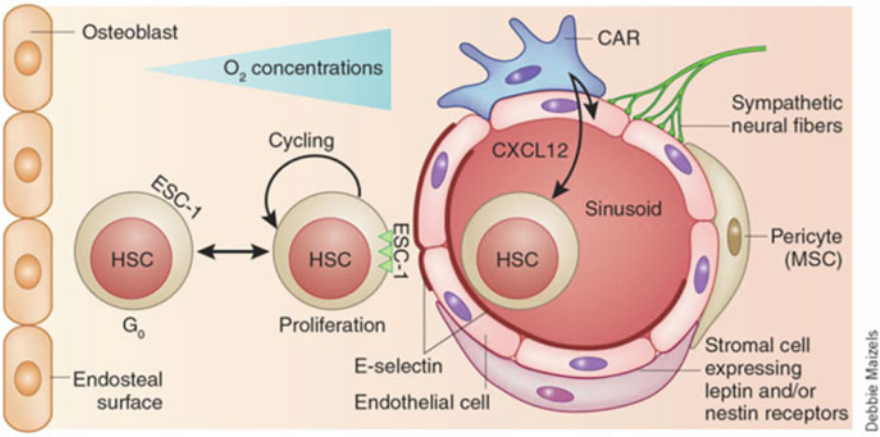

Fig. 7 HSCs in the bone marrow niche. The endothelial sinusoid is surrounded by MSCs identified as pericytes and a stromal subpopulation that can be identified by their expression of nestin and/or leptin receptors. CXCL-12-abundant reticular (CAR) cells are MSCs characterized by high expression of the chemokine CXCL-12, which is a chemoattractant for CXCR-4-expressing HSCs. Sympathetic neural fibers also connect with the niche and may provide negative proliferative signals. E-selectin is expressed exclusively on the endothelial cells of the niche and binds HSCs expressing appropriate ligand. ESC-1 is shown here as a candidate ligand, the binding of which to E-selectin is required for circulating HSCs to attach and roll on the sinusoidal endothelium before their firm attachment and egress. The attachment to E-selectin-expressing endothelium induces HSC proliferation, self-renewal, and chemo- and radiosensitivity. HSCs also reside close to the bone endosteal surface (a more hypoxic environment) and may receive instructive cues from osteoblasts and osteoprogenitors. (Moore 2012, with permission)

It is a complex structure bordered by bone and blood vessels. It is further composed of sympathetic neural fibers, perivascular cells including CAR (CXCL-12-abundant reticular cells), and pericytes. The latter two have MSC-like properties. As noted in Fig. 7, MSCs mediate HSC quiescence and differentiation likely through contact and paracrine signaling. Similar cells have been identified outside the marrow. There is some controversy as to nomenclature and whether cells isolated outside of the marrow are the same. Be that as it may, the minimal criteria for human MSCs noted in Table 2 have been quite useful in characterizing this stromal stem cell (Dominici et al. 2006).

Investigators have proposed that MSCs may be an ideal cell to examine tissue regeneration strategies. As noted earlier, they are readily isolated, large numbers of cells can be generated in culture, and they are thought to downregulate immune reactivity, perhaps as a consequence of their role in maintaining HSC quiescence. Following IUT in sheep, two groups have demonstrated human MSCs' migration to various organs and evidence for relevant (organ-based) differentiation (Liechty et al. 2000; Almeida-Porada et al. 2001). Rather than investigating late MSC engraftment/expression, another study investigated early events following transplantation of human fetal liver-derived MSCs in utero. At time points from 20 to 120 h following transplantation, the liver, lung, and brain were assessed for human cell presence using a labelled marker for the human cells.

A progressive expansion of the number of human MSCs was seen in each organ (Table 3). The high number of human MSCs in the lung was likely over-represented by lung entrapment. Staining for proliferation with anti-Ki67 was seen in labelled human cells. Site-specific differentiation of the cells was noted for each respective organ. Thus, for example, liver differentiation was seen in the liver but not the lungs. FISH analysis failed to detect fusion or membrane transfer with host/recipient sheep cells (Colletti et al. 2009).

As noted above, MSCs have been identified in multiple organs based on minimal criteria for human MSCs. The interest in endocrine pancreatic regeneration prompted a study using human fetal pancreatic MSCs. Pancreatic tissue was digested with collagenase and placed in initiation medium, and then the adherent cells were placed in a restrictive medium to eliminate contaminating cells in cell suspensions. Analysis of the recovered cell population revealed the requisite surface phenotype and in vitro differentiation into osteoblasts, adipocytes, and chondroblasts. The gene expression profile resembled marrow-derived MSCs rather than characteristics of pancreatic stem/progenitor cells.

The derived MSC population was transplanted in utero into fetal sheep and analyzed for multi-lineage differentiation and pancreatic engraftment. In addition, differentiation and engraftment of human hemopoietic elements were identified in the marrow, and human hepatocytic engraftment was noted with the secretion of human albumin.

Three months following transplantation, three of four sheep pancreas were noted to contain the human GADPH gene. Subsequent analysis was performed at 7, 25, and 27 months following transplantation. Using real-time quantitative polymerase chain reaction (PCR), human DNA was detected in the pancreatic tail in eight of ten transplanted sheep. It is conceivable that sampling error could contribute to the absence of human DNA detected from the four samples collected from each pancreatic tail. Functional expression was tested by analyzing the above ten sheep for the presence of human C-peptide (human proinsulin) in the circulation at 7, 25, and 27 months post-transplant. All animals were fasting; five of the animals that were chimeric based on PCR testing also tested positive for circulating C-peptide on at least two time points. Also, immunohistochemical studies localized clusters of pancreatic cells expressing human insulin (Ersek et al. 2010). In a similar fashion, Pixley's research group has detected in situ human insulin in the sheep pancreas following IUT using bone marrow-derived human MSCs (Fig. 8, Pixley 2020).

Stem Cell Plasticity

Thus, various research groups have described differentiation patterns of SCs in vitro and in vivo that contradicts the "well-established" concept that somatic stem cells do not cross lineage boundaries, for example, mesoderm lineage to endoderm lineage. Indeed, examples exist that SC differentiation can cross all lineages. For instance, following clinical bone marrow transplantation for malignant disease, donor hepatocytes and epithelial cells have been identified (Körbling et al. 2002). Most models testing this hypothesis have used tissue injury to promote SC "plasticity," yet IUT

Table 3 Percentage of engraftment and total number of human MSC detected in each organ at various times post-transplantation

		Liver			Lung			Brain		
Hours post-Tx	No. of animals	% Engraftment	Total no. of MSC	Total no. of Ki67*	% Engraftment	Total no. of MSC	Total no. of Ki67*	% Engraftment	Total no. of MSC	Total no. of Ki67*
20	3	0	0	0	0	0	0	0	0	0
25	3	0.0334 ± 0.0073	1.16 ± 0.008 × 10^5	1.1 ± 0.0 × 10^5	0	0	0	0	0	0
30	3	0.0621 ± 0.0061	2.15 ± 0.01 × 10^5	2.1 ± 0.0 × 10^5	0.0289 ± 0.0073	13.45 ± 0.09 × 10^5	12.9 ± 0.0 × 10^5	0	0	0
40	10	0.1343 ± 0.0244	4.64 ± 0.11 × 10^5	4.5 ± 0.1 × 10^5	0.0668 ± 0.0089	31.02 ± 0.28 × 10^5	29.5 ± 0.1 × 10^5	0.0340 ± 0.0078	1.71 ± 0.01 × 10^5	1.6 ± 0.0 × 10^5
60	10	0.1045 ± 0.0154	3.61 ± 0.06 × 10^5	3.5 ± 0.1 × 10^5	0.0681 ± 0.0224	31.61 ± 0.69 × 10^5	30 ± 0.0 × 10^5	0.0795 ± 0.0064	4.00 ± 0.02 × 10^5	3.8 ± 0.0 × 10^5
120	7	0.1041 ± 0.0155	3.96 ± 0.06 × 10^5	3.8 ± 0.0 × 10^5	0.0974 ± 0.0150	45.18 ± 0.67 × 10^5	42.5 ± 0.1 × 10^5	0.0652 ± 0.0157	3.29 ± 0.05 × 10^5	2.9 ± 0.0 × 10^5

Colletti et al. (2009), with permission
*Marker of cell proliferation

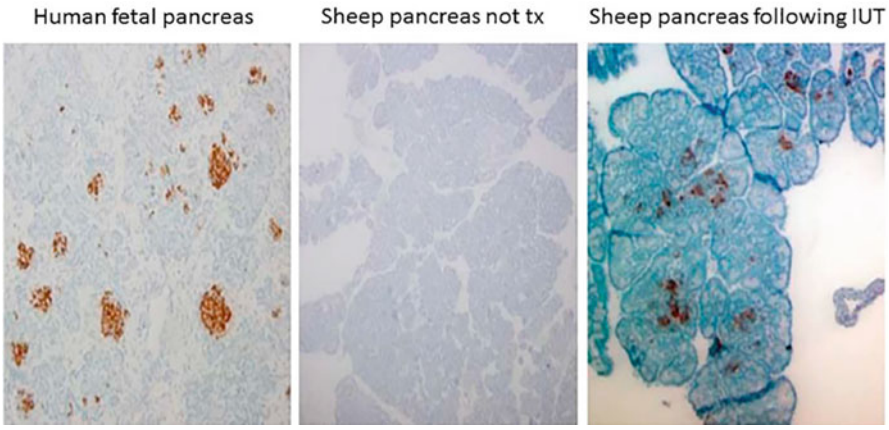

Fig. 8 Sheep pancreatic cells contain human insulin following IUT with human bone marrow-derived MSC. 5×10^5 human BM-MSC transplanted into a day 60 gestational age sheep fetus. Note pancreatic cell clusters which stain with a specific antibody to human insulin 4 months after transplantation. Human fetal pancreas, positive control; non-tx sheep pancreas, negative control. (Pixley 2020, with permission)

does not rely on an injury signal to cause SCs to "cross lineage boundaries." In part, this may be due to the achievement of complete transplantation tolerance. An alternate or additional possibility for these observations is that the fetal environment is permissive to hepatocytic and endocrine pancreatic differentiation. This suggests that developmental signals in, for example, the fetal sheep pancreas or liver can promote functional differentiation of human SCs to the endocrine pancreas or liver.

Lineage-restricted assays of hemopoietic expression have suggested that the graft can be expanded after birth (due to the achievement of immune tolerance) (Peranteau et al. 2002; Almeida-Porada et al. 2007). However, presently, no studies have reported that test graft expansion following IUT in organ regeneration in vivo after birth.

Extensive controversy with the above has arisen as well. This has been aptly summarized by Theise in several publications (Theise 2003, 2006, 2010). Specifically, the inability to reproduce hepatic and endocrine pancreatic engraftment and expression has been attributed to methodologic differences among the experiments. Also, reports of fusion as an explanation for differentiation into mature lineages suggested to some that true plasticity does not occur. However, others are less convinced that fusion discounts plasticity and believe it is one of many modes of cell plasticity (Theise 2010; Quesenberry et al. 2010).

Some reticence to accept these findings is the crucial role of immunohistochemistry and FISH analysis using intact fixed tissue samples in demonstrating donor cell expression. In most of the experiments, Pixley and colleagues were able to confirm human engraftment often years after transplantation by identifying human DNA fragments in tissue using PCR and demonstrated functionality by detecting donor-specific circulating proteins in the circulation of animals with immunohistochemical evidence of tissue engraftment (Table 4).

Table 4 Human proteins detected in circulation of animals transplanted in utero with human stem cells

Animal transplanted	Human protein detected
Sheep	IgM
Sheep	Factor VIII
Sheep	C-peptide
Sheep	Albumin
Sheep	Alpha-fetoprotein
Mouse	IgM

Pixley and Zanjani (2013), with permission

As noted, SC plasticity-related research is highly dependent on expertise in tissue histology and staining. Several methodologic concerns have been raised to ensure accurate reporting. For example, evidence of lung epithelial derivation using bone marrow cells is hampered by the lack of a functional marker. Thus, for identification in the lung of these rare donor bone marrow-derived epithelial cells, it is crucial that:

1. A confocal or deconvolution microscope is used
2. Marrow-derived epithelial cells are not just identified by morphology but also stained for a lung epithelial cell marker not present on bone marrow cells
3. High-quality high-magnification images are presented
4. Proper positive and negative controls are used

Effects requiring proper controls include artifacts, autofluorescence, false-positive signals, non-specific staining, overfixation, and lack of demonstration of specific staining on positive controls (Kassmer and Krause 2010).

What Is Stem Cell Plasticity, and How Can It Be Explained Physiologically

To answer this question, it is imperative to go back to two issues discussed previously. One, SC populations are heterogeneous as Zanjani and colleagues, among others, have demonstrated. Attempting to use phenotypic markers [in general, cell phenotype is identified by light scatter characteristics and labelling with fluorescent antibodies to surface antigens (CD34, e.g.)] will only "isolate" a subpopulation that possesses SC characteristics (self-renewal) identified using serial transplantation or temporal length of engraftment/expression capability and multi-lineage differentiation. The phenotype identified may only represent a cell at a particular phase of the cell cycle (Quesenberry et al. 2010). As observed, CD34 expression varied in serial transplantation studies. Indeed, lineage-negative (Lin−) cells appear to represent the most primitive SC "phenotype" based on engraftment, expression, and exhaustion testing. Secondly, revisiting Fig. 7, the representation of the bone marrow niche, it is obvious that the HSC in two phases based on ligand binding, in either G_o or cycling phase. While it is recognized that some of these cycling cells differentiate into the various hemopoietic lineages, some enter the circulation and populate various organs. Similarly, MSCs are found to circulate and lodge in other organs. It is thought these cells provide a reparative function, although the sheep non-injury

model suggests there may be additional explanations. For example, as suggested by Theise, is the lineage boundary paradigm a myth? Since it appears that tissue-based SCs are physiologic, peripheral differentiation capabilities appear to be much closer to those of embryonic SCs than previously thought (Theise 2006; Ratajczak 2015).

Epigenetics is any heritable influence (in the progeny of cells or individuals) on gene activity, unaccompanied by a change in DNA sequence. Thus, epigenetics is the study of the chromosome. Recent methodologic advancements have improved the understanding of gene regulation via not only DNA methylation and histone modifications but also the importance of the external environment and nuclear architecture in determining cell fate. Recent studies point to a stochastic model in cell fate decisions (Holliday 2006; Kærn et al. 2005; Cremer and Cremer 2001; Bornfleth et al. 1999; Merrell and Stanger 2016; Cosgrove and Wolberger 2005; Sun et al. 2020).

Historically, terms like transdifferentiation have been used to explain the cross-lineage differentiation of SCs. Yet SCs normally migrate to the sites distant from the marrow and differentiate based on location (in the context of immune tolerance). As these cells are immature, the stochastic model allows for differentiation based on the external environment without invoking transdifferentiation as an explanation. Thus, immature SCs/progenitors migrate, and their fate is mediated by what is in the immediate vicinity not internal factors; this coupled with the random motion of subchromosomal foci in the cell nucleus in part explains stochastic "regulation" of gene expression. "It is consistent with a topological model of gene regulation" (Cremer and Cremer 2001). As a corollary, bone marrow SCs differentiate to hemopoietic elements not based on predetermined chromatin-derived regulation but due to cell contact and paracrine signaling present in the bone marrow environment that support differentiation into the various hematopoietic lineages.

In summary, analysis of human SC migration, homing, and differentiation following IUSCT supports the concept that these cells are pluripotent, capable of extensive multi-lineage expression in vivo with accompanying functional relevance based on the presence of human proteins in sheep circulation years after transplantation (Table 4). Interestingly, Ray Owen used the term "embryonal ancestral cells" to describe his clearly prescient observation 76 years ago.

IUT as a Clinical Option

As noted earlier, early IUT studies in mice and sheep were conducted with the long-term goal of treating fetal diseases. The capability to test the fetus for the presence of these diseases early in gestation allows adequate time for IUT to be performed prior to the closure of the engraftment window. This period as discussed earlier is characterized by the developmental events: vascularization and formation of the thymic medulla (Maddox et al. 1987; Ohki et al. 1987; Laufer et al. 1999; Naquet et al. 1999). [The medulla is thought responsible for deletional and cellular tolerance.] It is known that thymic epithelial progenitor maturation is critically dependent upon interaction with dendritic cells (DC). Therefore, performing the procedure when peripheral DCs can migrate to the newly vascularized thymic medulla and

present donor antigen(s) to the maturing epithelial progenitors is thought responsible for establishing the tolerance repertoire (Pixley and Zanjani 2013; Fairchild and Waldmann 2000; Rodewald et al. 2001). Evidence supports the establishment of both deletional and cellular tolerance to donors following IUT.

At present, there is at least one preliminary clinical trial of IUT for thalassemia (MacKenzie 2021). Diseases thought amenable to IUT include hemoglobinopathies, immunodeficiency states, and inborn errors of metabolism leading to storage diseases (mucopolysaccharidosis and mucolipidosis). Hesitancy to move forward in the clinical arena has been primarily based on evidence for an immune barrier following allogeneic IUT in mice. For example, there is evidence that maternal immune cells or antibodies either in milk or following trans-placental transfer are responsible for some examples of maternally derived graft failure in mice (Merianos et al. 2009; Nijagal et al. 2011). Others reported again in mice that long-term chimerism requires an engraftment threshold whereby low levels of early engraftment shortly after birth are lost over time. Here the cells thought responsible are natural killer cells (Alhajjat et al. 2015). An alternative concern is in performing IUT in a host without a specific lineage defect. Here, the donor graft may be unable to expand due to competition from endogenous host cells.

To answer this question, Flake and colleagues performed a series of studies using dogs as hosts. Due to concern for maternally derived inhibition, maternal SCs were used as donor cells. Also, rather than an intraperitoneal injection, the SCs were transplanted using the intravascular route. Impressive chimeric levels were seen in most of animals transplanted via the vascular route (Vrecenak et al. 2014). Interested readers are referred to several recent publications for more detail (Vrecenak and Flake 2013; Derderian et al. 2015; Almeida-Porada and Porada 2019).

Conclusion

The exponential expansion in the general understanding of stem cell biology offers promise with regard to regenerative medicine. As noted above, this research has revealed a number of surprises, most notably the pleiotropic or pluripotent potential of these rare undifferentiated cells. In addition, in utero transplantation with the achievement of complete transplantation tolerance coupled with performing the procedure in large animals has provided confirmation and expansion of knowledge derived from smaller animals and in vitro studies. Thus, moving forward, large animal research should be an integral component in the armamentarium of methods to study stem cell biology.

Cross-References

- ▶ Human Mesenchymal Stem Cells: The Art to Use Them in the Treatment of Previously Untreatable
- ▶ Mesenchymal Stem Cells
- ▶ Sources and Therapeutic Strategies of Mesenchymal Stem Cells in Regenerative Medicine

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Part III

Cell-Free Therapy Approach

Mesenchymal Stem Cell-Extracellular Vesicle Therapy in Patients with Stroke

32

Oh Young Bang, Eun Hee Kim, Gyeong Joon Moon, and Jae Min Cha

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O. Y. Bang (✉)
Department of Neurology, Samsung Medical Center, School of Medicine, Sungkyunkwan University, Seoul, South Korea

S&E Bio, Inc, Seoul, South Korea
e-mail: ohyoung.bang@samsung.com

E. H. Kim
S&E Bio, Inc, Seoul, South Korea
e-mail: micro1109@snebio.com

G. J. Moon
AMC Stem Cell Center, Asan Medical Center, Seoul, South Korea

Department of Convergence Medicine, University of Ulsan College of Medicine, Seoul, South Korea
e-mail: gj.moon@amc.seoul.kr

J. M. Cha
Department of Mechatronics Engineering, College of Engineering, Incheon National University, Incheon, South Korea

3D Stem Cell Bioengineering Laboratory, Research Institute for Engineering and Technology, Incheon National University, Incheon, South Korea
e-mail: j.cha@inu.ac.kr

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Abstract

Stem cell-based therapy is a promising approach for treating acute stroke patients and stroke survivors with fixed neurological deficits. Several stem cell trials conducted in stroke patients have reported inconsistent results. Stem cells such as mesenchymal stem cells (MSCs) secrete extracellular vesicles (EVs), which harbor several molecules such as proteins and microRNAs. Recently, many preclinical studies have shown that stem cell-derived EVs can be used in stroke therapy as an alternative approach to stem cell application. This study discusses the evidence regarding the effects and underlying mechanisms of EV therapy in experimental stroke and findings of the biomarker sub-study from a randomized control trial of MSCs-based therapy in stroke patients. Moreover, the advantages and disadvantages of EVs therapy are compared with those of MSC therapy for stroke. Finally, major issues in the clinical application of EV therapeutics in stroke are discussed with relevant advances for clinical-scale EV production/enrichment, isolation/purification, and quantification/characterization. Several methods to improve the efficacy and purity of EV products have been introduced recently. This review presents the most recent advances in MSC-derived EV therapy for stroke, focusing on the application of this strategy in patients with ischemic stroke.

Keywords

Stroke · Extracellular vesicles · Exosomes · Stem cells · Ischemic stroke

List of Abbreviations

BBB	Blood–brain barrier
CDN	Cell-derived nanovesicles
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GMP	Good Manufacturing Practice
iPSCs	Induced pluripotent stem cells
ISEV	International Society for Extracellular Vesicles
lncRNAs	Long noncoding RNAs
MiRNA	MmicroRNAs

MISEV	Minimal Information for Studies of Extracellular Vesicles
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
NSCs	Neural stem cells
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
VEGF	Vascular endothelial growth factor

Introduction

Stroke is the leading cause of physical disability among adults. It is also the leading cause of death in developing countries and the third most common cause of death in most developed countries. Stem cell-based therapy is a promising approach for treating acute stroke patients and stroke survivors with fixed neurological deficits. However, several stem cell trials conducted in stroke patients have reported mixed results (Bang et al. 2016).

The mechanism of action of stem cells such as mesenchymal stem cells (MSCs) is the secretion of trophic factors and extracellular vesicles (EVs). EVs, circular membrane fragments measuring 30 nm–1 μm, contain cellular proteins, DNAs, RNAs, and mitochondria. EVs refer to a heterogeneous group of vesicles released from cells. They have been classified as exosomes and microvesicles (MVs) based on biogenesis. Exosomes are small EVs (30–100 nm) derived from multivesicular bodies, whereas MVs or ectosomes are large EVs (100–1,000 nm) that bud directly from the cellular membrane into vesicles. According to the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines, EVs can be classified as small EVs (<200 nm) and medium or large EVs (>200 nm) based on the size of the particles because the subcellular origin may not be determined (Thery et al. 2018).

Like any other cell type, EVs are inherently released by stem cells as part of their paracrine activity and they have shown great promise in clinics as an innovative cell-free therapy. They inherently possess the properties of essential mediators of regenerative activities and tissue homeostasis in vivo. MSCs-derived EVs (MSC-EVs), as the substitute for the current MSC therapy, exhibit the following advantages: first, MSC-EVs contain numerous therapeutic biomolecules released by MSCs. EVs harbor bioactive molecules. Moreover, EVs secreted by stem cells carry more complex cargos than those secreted by other cells (Lai et al. 2012). MSC-EVs contain many molecules that may have therapeutic effects against stroke, such as RNAs, proteins, lipids, and mitochondria (Katsuda and Ochiya 2015). Therefore, unlike sole molecules (such as synthesized small chemicals) or macromolecules (such as protein and antibodies), EVs contain payload of heterogeneous biomolecules and are considered more appropriate tools for treating various diseases. Second, EVs exhibit many benefits concerning biocompatibility, immunogenicity, stability, pharmacokinetics, biodistribution, and cellular uptake mechanism (Nam et al. 2020). Their nano-sized, lipid-shielded vesicular structure would be safer and favorable for long-term circulation in the blood and long-distance therapeutic actions

than MSCs; also, EVs bearing MSC-specific membrane proteins on their surfaces could potentially confer disease-targeting ability as infused MSCs (Biancone et al. 2012; Karp and Leng Teo 2009).

Advantages of EVs over MSCs in Stroke

EVs have low toxicity, high stability in the circulation; advantages in scalable production and storage, and high transport efficiency (can pass the blood–brain barrier (BBB) and avoid the first-pass effect). First, the cell-related problems of MSCs-based therapy can be avoided by using this cell-free paradigm (i.e., MSC-EVs). The diameter of MSCs is large (15–30 μm) that leads to passive arrest of MSCs in small-diameter vessels, causing vascular occlusion and reduction in cerebral blood flow when administered intra-arterially and entrapment in systemic vessels in organs such as the lungs when administered systemically (the first-pass effect) (Krueger et al. 2018; Pendharkar et al. 2010; Nguyen et al. 2016). High mortality following intravenous transplantation of MSCs in experimental animal models of stroke and familial occurrence of pulmonary embolism/infarct after intravenous injection of allogeneic adipose-derived MSCs have been reported (Jung et al. 2013). The MSCs-related pro-coagulation status could explain such lethal pulmonary thromboembolism (Tatsumi et al. 2013). On the other hand, MSC-EVs capable of crossing the BBB in both directions (Nam et al. 2020) may alleviate the cell-related problems of stem cell-based therapy, i.e., vascular occlusion, mortality, and the first-pass effect (Moon et al. 2019).

Second, allogeneic MSC-EVs' scalable production allows their "off-the-shelf" availability to treat acute ischemic stroke patients. Regarding EVs stability, Kalra et al. showed that EVs retain their integrity for 3 months when stored at 37 °C, 4 °C, −20 °C, or −80 °C (Kalra et al. 2013), while Sokolova et al. reported that multiple freeze-thaw cycles did not affect the quality of MSC-EVs if stored at −20 °C in phosphate-buffered saline (Sokolova et al. 2011). Thus, both neurorestorative and neuroprotective actions can be expected using MSCs-EVs based approach in the acute phase of stroke (Savitz 2013). In addition, the use of allogeneic MSC-EVs has some additional benefits. The source of EVs is an essential determinant of their efficacy in stroke. MSCs from elderly patients have limited restorative potential and hence, MSC-EVs derived from the aging MSCs may have age-dependent and aging-relevant differences in their cargo contents compared to their healthy juvenile and young counter-parts (Fafian-Labora et al. 2017). Similarly, allogeneic MSC-EVs from the umbilical cord may differ from those obtained from elderly stroke patients with other chronic illness in terms of their proliferation and neurorestorative capacity (Fafian-Labora et al. 2017; Eirin et al. 2020; Li et al. 2020). It is interesting to note that transfer of EVs from young MSCs to aged stem cells resulted in their rejuvenation (Kulkarni et al. 2018). Fetal MSCs derived from amniotic fluid-, cord blood-, and Wharton's Jelly have intermediate cellular phenotypes between embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), and adult stem cells such as bone marrow MSCs in terms multi-potency and expression patterns of both surface markers and/or transcription factors of pluripotency and mesenchymal commitment

(Loukogeorgakis and De Coppi 2017). Although ESC/iPSC-derived EVs-based therapy may be safer than ESC/iPSC cell-based therapy in terms of tumorigenicity, limited data are available in the field of stroke and human trials (Webb et al. 2018b; Kalani et al. 2016). Therefore, fetal MSCs may be a good source of EVs for clinical applications. Similarly, systemic diseases may determine the efficacy of MSC-EVs in stroke. The payload of MSC-EVs from obese donors is different from those obtained from non-obese donors (Eirin et al. 2020; Li et al. 2020).

Evidence of the Role of Stem Cell-Derived EVs in Stroke

The effects of EV-based therapeutics have been reported in various experimental animal disease or injury models (Fuster-Matanzo et al. 2015; Cunningham et al. 2018; Doeppner et al. 2018). Many preclinical studies have recently shown that stem cell-derived EVs can be used in stroke therapy (Bang and Kim 2019).

Preclinical Evidence of EV Therapeutics in Stroke Models

In 2013, Xin et al. were the first to report that intravenous injection of MSC-EVs in a rat model of stroke improved the neurological outcomes and increased angiogenesis and neurogenesis (Xin et al. 2013). Other investigators have also demonstrated the beneficial effects of stem cell-derived EVs in various animal models of stroke (Table 1). Several advances in the EVs-based strategy have been introduced, which are as follows:

(a) Use of EVs from stem cells other than MSCs, such as ESCs, neural stem cells (NSCs), and iPSC-derived MSCs or NSCs (Webb et al. 2018a, b; Kalani et al. 2016).
(b) Application of EVs through the intranasal approach (Kalani et al. 2016).
(c) Production of EVs using 3D dynamic culture method, to increase the production and regulate the payload of EVs (Cha et al. 2018b) and stimulation with ischemic brain extracts (Moon et al. 2019; Lee et al. 2016).
(d) The use of various EV isolation methods other than ultracentrifugation (Doeppner et al. 2015; Chen et al. 2016; Otero-Ortega et al. 2017).

Recently, EVs' effects on stroke have been evaluated in large animal (i.e., pigs and monkeys) models of stroke (Webb et al. 2018a; Medalla et al. 2020).

Clinical Evidence of EV Efficacy in the Clinical Trial of MSCs in Stroke Patients

Our research group has recently reported the results of the Stem Cell Application Researches and Trials In Neurology-2 trial, a randomized controlled trial of intravenous application of autologous MSCs expanded with autologous serum

Table 1 Applications of stem cell-derived extracellular vesicles in stroke in various experimental animal models

Reference	Animal Model	Stem cells/mode of application	EV production/culture media	EV isolation/dose per animal	Major findings
Xin et al. 2013	Rat	Rat BM MSCs/intravenous	2D culture/exosome-free serum	Ultracentrifugation/100 μg total exosome protein	Angiogenesis; neurogenesis Neurological recovery
Doeppner et al. 2015	Mice	Human BM MSCs/ intravenous	2D culture/MSC basal media	PEG/EVs released by 2×10^6 cells	Neuroprotection; angiogenesis Neurogenesis; immunomodulation Neurological recovery
Chen et al. 2016	Rat	Mini-pig adipose MSCs/ intravenous	2D culture/10% fetal bovine serum	KISO™ system/100 μg total exosome protein	Neuroprotection Neurological recovery
Kalani et al. 2016	Mice	Mice ESCs/intranasal	2D culture on fibroblast monolayer/exosome-free serum	Ultracentrifugation/NA	Restoration of neurovascular unit Immunomodulation
Otero-Ortega et al. 2017	Rat	Rat adipose MSCs/ intravenous	2D culture/exosome-free serum	Exosome extraction kit (miRCURY)/ 100 μg total exosome protein	Neuroplasticity; white matter recovery Neurological recovery
Xin et al. 2017a	Rat	Rat BM MSCs/intravenous	2D culture/exosome-free serum	Ultracentrifugation/100 μg total exosome protein	Neuroplasticity; neurological recovery
Xin et al. 2017b	Rat	microRNA-133b overexpressing rat BM MSCs/ intra-arterial	2D culture/exosome-free serum	Ultracentrifugation/3×10^{11} EVs, comparable to 100 μg total exosome protein	Neuroplasticity Neurological recovery

Nalamolu et al. 2019	Rat	Human UCB MSCs	2D culture /exosome-free serum	Total exosome isolation kit/150 μg total exosome protein	Infarct size reduction Adverse effects on recovery
Wang et al. 2020	Rat	Human BM MSCs from 3 donors/ intravenous	2D culture/hypoxia or normoxia culture media	PEG/EVs obtained from 2×10^6 MSCs,	Neuroprotection; immunomodulation BBB permeability
Lee et al. 2016	Rat	Human adipose MSCs/ intravenous	2D culture/serum free media with brain extract	Ultracentrifugation/0.2 mg/kg	Angiogenesis; neurogenesis Immunomodulation
Moon et al. 2019	Rat	Rat BM MSCs/intravenous	2D culture /serum free media with brain extract	Ultracentrifugation/30 μg total exosome protein	Angiogenesis; neurogenesis Neuroplasticity; neurological recovery
Cha et al. 2018b	In vitro	Human BM MSCs	3D dynamic culture /serum free media	Ultracentrifugation/NA	Angiogenesis; neurogenesis Neurological recovery
Webb et al. 2018a	Pig	Human NSCs/intravenous	2D culture /NSC basal culture media	Ultracentrifugation/2×10^{10} EVs/kg	Neuroprotection Neurological recovery
Medalla et al. 2020	Monkey	Adult monkey BM MSCs	2D culture/exosome-free serum	Ultracentrifugation/4×10^{11} EVs/kg	Neuroplasticity
Webb et al. 2018b	Mice	iPSC-derived NSCs or MSCs/ intravenous	NA	NA	Neuroprotection; immunomodulation Neurological recovery

Abbreviations: *EV* extracellular vesicles, *2D* two-dimensional, *3D* three-dimensional, *BBB* blood-brain barrier, *BM* bone marrow, *ESCs* embryonic stem cells, *iPSC* induced pluripotent stem cell, *MSC* mesenchymal stem cells, *NA* not available, *NSCs* neural stem cells, *PEG* polyethylene glycol precipitation method, *UCB* umbilical cord blood, *WJ* Wharton's jelly

(Chung et al. 2021). Although intravenous autologous MSCs' transplantation was safe in stroke patients, the beneficial effects were diverse among patients. The pre-specified biomarker sub-study showed that circulating EVs markedly increased after MSCs' injection, especially in patients who showed clinical improvement after MSC therapy, and these EVs harbored therapeutic molecules (Bang et al. 2022). Only the number of circulating EVs after MSCs' injection was independently associated with motor function improvement, as assessed by clinical and multimodal magnetic resonance imaging (MRI). These data have increased the possibility of the use of MSC-EVs, instead of MSCs per se, given that the number of EVs determines the effects of MSC-based therapy.

Status and Limitations of EV Therapeutics

Preclinical Studies

A systemic review of the preclinical studies on the therapeutic effects of MSC-EVs has shown that despite MSC-EVs demonstrated benefits in 97% of studies in various experimental disease models, there were several critical methodological concerns. These concerns included: the diversity in characterization and isolation techniques, dosing (protein vs. EVs concentration), the use of xenogeneic EVs, and an apparent lack of EVs biodistribution study post-delivery (Tieu et al. 2020). For example, approaches for determining size, protein markers, and morphology were highly heterogeneous with only 12 and 4 studies among 206 studies meeting the MISEV 2014 and 2018 recommendations, respectively. To minimize the sources of deviation, Kennedy et al. recommended scalable techniques for EV isolation, classical dose-response, optimal timing, repeat dosing experiments, use of inert (biologically removed) EVs control, and multiple analysis platforms such as proteome and transcriptome analyses (Kennedy et al. 2021).

Clinical Studies

Only a few clinical studies have reported the effects of EVs-based therapy in patients. Kordelas et al. reported a case study of refractory graft-versus-host disease treatment with allogeneic MSC-EVs (Kordelas et al. 2014). The authors used allogeneic MSCs' conditioned medium and EVs were isolated using the polyethylene glycol (PEG) precipitation method. EVs obtained from 4×10^7 MSCs were administered repeatedly for four times, which alleviated the clinical symptoms without adverse effects. In a study by Katagiri et al. locally injected allogenic MSC-EVs in eight patients requiring bone augmentation before dental implant placement and observed that this method was safe, and had tremendous osteogenic potential for alveolar bone regeneration (KatagirI et al. 2016). On the same note, Zhang et al. administered MSC-EVs by intravitreal injection to five patients with refractory macular holes (Zhang et al. 2018). Finally, Nassar et al. reported the

treatment safety and possibility of improving kidney function in patients with chronic kidney disease after the administration of allogeneic MSC-EVs (Nassar et al. 2016). All four clinical studies were small case series. Although these data suggest that MSC-EVs were safe and might improve prognosis, randomized trials investigating the safety and efficacy of the MSC-EVs therapy are required. Three clinical trials on allogeneic cord tissue-derived MSC-EVs are ongoing in patients with diabetes mellitus (clinicalTrial.identifier@gov: NCT02138331), macular degeneration (NCT03437759), and ischemic stroke (NCT03384433).

Considerations for the Application of EV Therapeutics in Stroke

Current Guidelines/Recommendations

Given that MSC-EVs are nano-sized non-self-replicating vesicles, the regulatory items required for the production of EV-fractions for clinical usage could be less complicated than those for MSCs-based therapy. However, clinical evaluation of EV-based therapeutics is at an early stage compared with MSC-based treatment. Current legislation in the USA and Europe does not provide specific regulation of EV-based therapies. Thus, EVs-based therapeutics are best categorized as "biological medicine" (a part of the biologics class for pharmaceutical development), a medicine that contains one or more active substances made by or derived from a biological cell; and multiple possible designations of EVs have different regulatory burdens (Fuster-Matanzo et al. 2015; Reiner et al. 2017). This pharmaceutical classification harbors unique challenges regarding pharmaceutical manufacturing and preclinical safety testing. Hence, additional studies are required to address the risk assessment concerns, i.e., characterization or safety tests of donor cells, purity/heterogeneity of EVs preparation, potency marker, therapeutic unit (e.g., RNA, protein, and their relationship to the mode of action), enrichment of harmful substances in EV preparations (e.g., virus), and quality assurance for manufacturing processes (Reiner et al. 2017). On the contrary, the documentation of trans-differentiation capacity of stem cells and in vivo safety assessment of EV therapeutics for tumorigenicity and immunogenicity may not be required.

Individual MSC-EV preparations may differ in their therapeutic efficacy based on the donor of MSCs and subtle changes during EV production and isolation. Wang et al. evaluated the therapeutic effects of MSCs from different healthy donors in an experimental animal model of stroke. They found that MSC-EV preparations of only some donors were able to reduce the infarct volume and neuronal injury. This raised the importance of potency assays to identify MSC-EV preparations' therapeutic efficacy before clinical use (Wang et al. 2020). Moreover, the characteristics and cargo of EVs are reported to vary based on sources (bone marrow, cord blood, adipose, and menstrual MSCs) and donors (Ragni et al. 2017; Lopez-Verrilli et al. 2016; Cai et al. 2020). Therefore, the results of one MSCs-EV preparation cannot be compared to other MSCs-EV preparations as the protocols and steps for manufacturing and characterization of EVs may differ besides the source of EVs and donors.

Several issues, i.e., specific guidelines targeting EVs-based therapeutics, characterization, isolation, and storage of EVs, quality control requirements, and in vivo analysis of EVs should be resolved before the clinical application of EVs. The International Society for Extracellular Vesicles (ISEV) has provided a minimal set of experimental requirements to attribute any specific biological cargo or functions to EVs (Lotvall et al. 2014), and the protocol and steps for EVs separation/enrichment, and EVs characterization to document-specific EV-associated functional activities were updated in 2018 (Thery et al. 2018). Although these issues have been discussed precisely in previous studies (Fuster-Matanzo et al. 2015; Lener et al. 2015; Reiner et al. 2017; Witwer et al. 2019), they deserve mention in the application of MSC-EVs for stroke patients.

Quality Control and Potency Markers

MSC-EVs should be defined by quantifiable parameters such as the size-distribution of EVs, presence of the markers of EVs (e.g., tetraspanins) and the lipid bilayer structure, the integrity of EVs, and the absence of contaminations (e.g., harmful substances, xenogeneic components, and non-EV proteins such as albumin and soluble acetylcholinesterase) and apoptotic bodies. Furthermore, several methods are available, and new protocols are being developed to quantify and characterize EVs (Hartjes et al. 2019).

The purity of EVs is of prime importance in the quality control of EVs-based therapeutics. Several methods have been used concomitantly for assessing EVs purity because each method has its advantages and disadvantages. The tests for EVs' purity include: the nanoparticle tracking analysis technique (NTA; involves direct observation of EVs in real-time, with the lower limit of the measurement of fluorescent particles being 30–40 nm, and is more reliable than protein-level assessments), EV particle number to protein ratio, CryoEM (direct observation of the morphological structure of EVs, including the lipid-bilayer), and ELISA and Western-blotting for quantification of EVs marker proteins (e.g., tetraspanins) and contaminating proteins (e.g., albumin).

The clinical challenge to the translation of EV therapeutics is to ensure that every batch of MSC-EVs released has the same potency. The potency is the specific ability or capacity of the products, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the product administration in an intended manner to affect a given result (Code of Federal Regulations, April 1, 2019). EVs have many therapeutic components and multiple modes of action; hence, potency and quality control markers should be carefully selected and measured during the storage and freeze-thaw. In stroke patients, potency markers of EV therapeutics may differ depending on the time (acute vs. chronic phase) of EV application. For example, acute ischemic stroke patients require EVs' cargo components targeting neuroprotection and immunomodulation. In contrast, both acute and chronic stroke patients require EVs' components targeting neurogenesis, angiogenesis, and synaptogenesis for neurorestoration. Differential markers for the potency of

EVs (in vitro bioassays) may be necessary for acute and chronic ischemic stroke patients. In addition, customization of stem cell-EV properties is required for stroke treatment.

RNAs

In the EV cargo, microRNAs (miRNAs) are of prime importance in mediating the therapeutic effects of MSC-EVs, whereas the role of EV proteins in recipient cells remains unclear (Zhang et al. 2019). MiRNAs are a class of short (20–25 nucleotides), single-stranded, non-coding RNAs that can be horizontally shuttled by EVs. The brain levels of miRNAs are changed after stroke, and miRNAs have been implicated in regulating protective and restorative processes in stroke (Saugstad 2010). Shojaati et al. performed a knockdown of Alix mRNA (Alix, a component of the endosomal sorting complex required for transport) using siRNA to reduce miRNA without changes in the protein level in the secreted EVs. They found that MSC-EVs reduced corneal fibrosis and inflammation via EVs-mediated miRNA delivery, emphasizing the role of miRNAs in EV functions (Shojaati et al. 2019). The mechanism of action of MSC-EVs in neurogenesis, angiogenesis, neuroprotection, and anti-inflammatory response in stroke involves several miRNAs (Bang and Kim 2019). For example, Xin et al. reported that intra-arterial administration of EVs obtained from miR-133b overexpressing MSCs to a rat model of stroke enhanced neuroplasticity and neurological recovery (Xin et al. 2017b). Current methods available for individual miRNA expression studies (e.g., PCR array and small RNA sequencing) have limitations quantitating EV-miRNAs, and miRNAs may be distributed in homogeneously across the EV population (Chevillet et al. 2014). New RNA analyzing methods may improve further understanding of miRNAs in EV effects. In addition to small noncoding RNAs such as miRNAs, MSC-EVs may shuttle other genetic components such as mRNAs or long noncoding RNAs (lncRNAs) (Ragni et al. 2017). lncRNAs (>200 nucleotides) control proteins targeting genomic loci and epigenetic silencing and serve as scaffolds for multiple proteins; stroke significantly alters the cerebral lncRNA expression (Dharap et al. 2012). Fan et al. showed that lncRNAs mediate stroke-induced neurogenesis (Fan et al. 2020). MSC-EVs may exert their action via lncRNAs (Patel et al. 2018). Besides, the transfer of EV mRNAs modulates the protein levels of recipient cells. mRNA and lncRNAs have roles in stroke pathophysiology and MSC-EV effects and warrant further studies (Fan et al. 2020).

Proteins

Lim et al. emphasized a protein-based mechanism of action of MSC-EVs (Lai et al. 2013; Toh et al. 2018). Many different laboratories have identified EV proteins using a mass spectrometry-based approach, suggesting that the protein composition of EVs is determined by various factors such as the type of cell and culture conditions. Data of over a thousand EV proteins are accessible on public online databases such as Vesiclepedia (Kalra et al. 2012) and Exocarta (Keerthikumar et al. 2016). EVs contain many membrane and intraluminal proteins, which are associated with various biological processes. Commonly, ESCRT-associated proteins such as Alix,

TSG101, HSC70 and HSP90, and tetraspanins (CD81, CD63, and CD9) are present in EVs. Therefore, these proteins are generally regarded as EVs' marker proteins independent of their cell source (Simpson et al. 2008; Doyle and Wang 2019). Other studies have reported that some proteins are commonly associated with EVs, which include transport proteins (Rab GTPases and annexins), signal transduction factors (kinases), metabolic enzymes, and cytoskeletal proteins (Simons and Raposo 2009; Chaput and Thery 2011). EVs protein analysis can also serve as an indicator of the purity and quality of EVs. Quality check of EV products is required for the clinical application of EV therapy. According to the updated ISEV experimental guidelines, the information of proteins in EVs obtained by protein-based EVs characterization is required to eliminate putative contaminants' tendency (Thery et al. 2018). Webber et al. suggested that high-purity EVs have a ratio of $>3 \times 10^{10}$ particles per μg of protein and low-purity EVs present a ratio of 2×10^{9} to 2×10^{10} particles per μg of protein (Webber and Clayton 2013). The EV particle-protein ratio could be changed according to culture conditions or isolation methods. 3D-culture can increase the EV release from a single cell but decrease the ratio of particles per μg of protein than 2D-culture (Haraszti et al. 2018; Cha et al. 2018b). Several groups have identified the proteome of MSC-EVs using various proteomic approaches. They have suggested that proteins in MSC-EVs have sufficient biochemical potency for disease pathogenesis or regenerative therapy (Xing et al. 2020; Lai et al. 2012; Kim et al. 2012; Angulski et al. 2017; Anderson et al. 2016; La Greca et al. 2018). Kim et al. characterized the MSC-EVs proteome and reported that EVs contain several candidate proteins that may mediate potential therapeutic effects such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibronectin, integrins, and various proteins associated with signaling pathways (Wnt, TGF-β, and RAS-MAPK pathways) (Kim et al. 2012).

Another group demonstrated that MSC-EVs contain growth factors i.e., glial cell-derived neurotrophic factor, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) and angiogenic factors (i.e., hepatocyte growth factor, Ang1, HES1, and S1P), which promote tissue repair and regeneration (Hu et al. 2015; Zhu et al. 2014). More recently, Xing et al. performed proteomic analysis of EVs released from adipose-derived MSCs and reported that the EV proteins participated in several enriched pathways such as the MAPK, VEGF, and Jak-STAT signaling pathways, which are related to tissue repair (Xing et al. 2020). Anderson et al. demonstrated that angiogenic signaling proteins in MSC-EVs increased when MSCs were exposed to ischemic conditions such as PDGF, EGF, FGF, and most notably nuclear factor-kappaB (NF-κB) signaling pathway proteins, suggesting that MSCs could release EVs containing robust pro-angiogenic paracrine effectors under pathological conditions (Anderson et al. 2016). Notably, some surface proteins of EVs, i.e., tetraspanins, integrins, extracellular matrix (ECM) proteins, immunoglobulins, proteoglycan, and lectins, play an essential role in the interaction of EVs with the plasma membrane of target cells. This interaction results in the EVs uptake or regulation of various signaling pathways in the target cells (Sheldon et al. 2010; Lam et al. 2020; Rana et al. 2012; Mulcahy et al. 2014).

Other Cargos

Lipids are essential molecular components of EVs because they make up the lipid bilayer membrane that protects the encapsulated material. However, information regarding the composition and function of lipids in EVs is limited. Membranous lipids have many roles, such as EV markers, trafficking or stabilizing EVs, transporting membranous lipids to recipient cells, transferring bioactive lipids or enzymes to recipient cells, and waste disposal (Skotland et al. 2020). Recently, Barzegar et al. showed that MSC-EVs promoted neuroprotective effects in an animal model of stroke in cholesterol or lipid-dependent manner (Barzegar et al. 2020). Several studies have suggested an association between mitochondrial dysfunction and brain impairments after stroke, and hence, transplantation of healthy mitochondria can be a promising approach in stroke (Hayakawa et al. 2018). MSCs control intracellular oxidative stress by targeting mitochondria. Wang et al. showed that EVs containing mitochondria were released from MSCs and engulfed by recipient cells by fusion (Wang et al. 2018a).

Isolation, Dosage, Mode of Application, and Biodistribution

In EV studies, two main dosing strategies used are the number of EVs and protein concentration. However, because protein concentration does not correlate with EV number readout (as assessed by NTA), this concentration is not valid for determining EVs dosing (Lobb et al. 2015). Different EVs isolation methods can yield samples with up to an eight-fold difference in protein content relative to EVs number from the same source material (due to co-isolating contaminating proteins) (Kennedy et al. 2021). Although the EV number yield can vary with isolation technique, it is more reliable than protein as a surrogate (Kennedy et al. 2021). However, it is observed that NTA, the most widely used method for measuring the EVs number, may show variation in EV numbers up to 25% (Vestad et al. 2017).

The optimal time and mode of EVs application should be studied in stroke patients. Most recovery occurs in the first few months following a stroke, with only minor additional measurable improvements occurring thereafter. The levels of chemokines, trophic factors, and related miRNAs increase markedly in the infarcted brain during the acute phase of stroke and decrease over time. Such changes in the brain microenvironment may significantly affect the biodistribution of EVs and the degree of recovery and neurogenesis/angiogenesis after administering EVs therapeutics in stroke patients.

The brain capillary endothelium forms BBB that prevents the passage of 100% of large-molecular neurotherapeutics and more than 98% of all small-molecular-sized drugs into the brain (Pardridge 2003). Hence, compared with the amount of MSC-EVs required to treat patients with topical diseases or other systemic illnesses with a local application, a more considerable amount of MSC-EVs may be required to treat stroke patients (especially in the chronic phase when the BBB is closed). BBB manipulation (e.g., with use of mannitol) may enhance endogenous repair

mechanisms following stroke by allowing entry of paracrine factors (e.g., trophic factors and EVs) more easily into the brain (Borlongan et al. 2004).

At 24 h after systemic application, EVs preferentially accumulate in the liver, spleen, and lung and at low but detectable levels in the kidney, heart, and brain. The average half-life of EVs in the circulation is short (between 2 and 20 min in mice) (Wiklander et al. 2015; Gudbergsson et al. 2019). The biodistribution of EVs is dose-dependent, and effected by the route of administration, and parent cell source of EVs (Wiklander et al. 2015). The biodistribution study using fluorescence-labeled MSC-EVs and MSCs in a rat model of stroke revealed that although most MSCs got trapped within the lung immediately after injection, the amounts of MSC-EVs in the infarcted hemisphere increased in a dose-dependent manner and were rarely found in the lung and liver overtime (Moon et al. 2019). Another analysis of EV treatment in a stroke rat model revealed that circulating EV levels did not differ with the dose (for the tested doses). Both low and high doses of EVs improved recovery after stroke (Otero-Ortega et al. 2020).

Production of EV Preparation

An optimal manufacturing process would have the following attributes: high capacity for mass production, closed system with defined disposable components, purity with high yield, serum-free cell culture conditions, and Good Manufacturing Practice (GMP) compliance (Reiner et al. 2017). Many different cell culture media are used to produce EVs, such as serum-supplemented media, serum-free media, and EVs-free/reduced serum-supplemented media. Prior elimination of EVs from fetal bovine serum is crucial; commercial exosome/EVs-depleted serum is expensive and maybe imperfect. Hence, various methods to deplete EVs are being investigated, such as the ultrafiltration method or the use of xeno-free and chemically defined media (Kornilov et al. 2018).

The heterogeneity of EVs and their cargo may increase or decrease during the production of EVs. Further in-depth studies are required to curtail the heterogeneity of EVs and increase the levels of the therapeutic components of EVs in clinically feasible ways for stroke patients (Xin et al. 2017b; Cha et al. 2018b; Wang et al. 2018b; Harting et al. 2018; Domenis et al. 2018). MSCs heterogeneity (donor variation) because of the origin of MSCs or the conditions of donors (e.g., age and diseases) can be minimized with optimal culture conditions or with the use of a working cell bank (Costa et al. 2021).

Isolation of Prepared EVs

Contamination of non-EVs material is by far the greatest variable associated with the isolation method, with significant potential for the non-specific effects of EV-based therapeutics. Moreover, EVs from stem cells exhibit heterogeneity in terms of size, and EVs of a different size may show differential effects when used as therapeutics. Differential EVs isolation methods can yield samples with varying degrees of

contaminating proteins and therapeutic cargo proteins/miRNAs/lipids, although the material source is the same.

Various techniques that include (but are not limited to) ultracentrifugation, PEG precipitation, size exclusion chromatography, and tangential flow filtration are available for the isolation of EVs. However, each method has advantages and disadvantages; hence, no reliable method for the isolation of EVs is available (Reiner et al. 2017). A recent survey performed by the ISEV revealed that ultracentrifugation and density-gradients are included in the most commonly used protocols for EVs isolation and purification. Moreover, size-exclusion chromatography and tangential flow filtration are being increasingly used recently (Royo et al. 2020). In addition, Watson et al. suggested GMP-compatible methods for clinical-scale production, purification, and EVs isolation (Watson et al. 2018).

Recent Advances in EV Therapeutics

The clinical translation of EVs-based therapeutics is impeded by some practical issues such as heterogeneity in both extensive therapeutic cargo and surface configuration, which could lead to uncontrollable or lower therapeutic efficacy and low yield. This may be problematic for a stable supply of potent EVs-based medicinal products, especially at the onset of phase III clinical trials and on the market scale (Gimona et al. 2017). To date, thanks to great strides in understanding complicated EVs physiology, numerous bioengineering methodologies are developed to address these challenges and help support the onward clinical advances. This section will discuss the current state-of-the-art bioengineering technologies that have been designed to augment the therapeutic potency and production yield of stem cell-derived EVs.

Since EVs are secreted products of cells, there have been various attempts in research to genetically or biochemically modulate parental cells' phenotypes to influence the yield and therapeutic potential of the resulting EVs. MSC-EVs have often been reported as an efficient promoter of angiogenesis, as they incorporate a multitude of angiogenic factors. Tao et al. reported that the proangiogenic ability of MSC-EVs is further enhanced by genetic modification of MSCs to overexpress miRNA-126, one of the primary angiogenic mediators both in vitro and in vivo (Tao et al. 2017). Kang et al. reported that EVs derived from MSCs overexpressing CXC chemokine receptor 4 significantly promoted angiogenesis in a rat model of myocardial infarction and protected neonatal cardiomyocytes apoptosis in vitro (Kang et al. 2015). In the stroke field, engineering MSCs to overexpress specific therapeutic proteins or RNAs increased their efficacy after stroke (Xin et al. 2017b). A wide variety of molecules and culture methods prime MSCs and modify their EVs accordingly. For example, preconditioning of sub-lethal stimuli can trigger an adaptive response of MSCs to injury or damage. Moon et al. showed that the cultivation of MSCs with either serum of stroke patients or treatment with ischemic brain extracts could increase MSCs' restorative properties and EVs release. These data suggested that signals from an ischemic brain could affect the efficacy of MSCs

and MSC-EVs while stimulating EVs release from MSCs (Moon et al. 2018, 2019). Similar findings were reported by Lee's research group (Lee et al. 2016). Treatment with ischemic brain extract and MSCs-conditioned medium upregulated miRNAs and proteins that modulate tissue repair pathways (Moon et al. 2019; Lee et al. 2016). Thrombin-preconditioning of MSCs increased EVs yield and enriched their therapeutic payload of interest (Sung et al. 2019). It is widely accepted that hypoxic culture conditions similar to the bone marrow microenvironment (i.e., 0.1–2% O_2) are beneficial to MSCs as they exhibit adaptive cell response to the injury sites. MSCs culture under hypoxic conditions with or without serum deprivation amplified the secretion of EVs, enriched therapeutic payload (e.g., miRNAs), and improved their efficacy in experimental tissue injury models (Zhang et al. 2012; Bian et al. 2014; Wang et al. 2018b, 2020; Park et al. 2018). Pro-inflammatory priming of MSCs renders EVs release with enhanced anti-inflammatory properties (Harting et al. 2018; Domenis et al. 2018; Hyland et al. 2020).

Exogenous supplementation of bioactive molecules (e.g., growth factors, cytokines, and chemicals) in MSCs culture could influence the cellular biosynthesis machinery, and thus, regulate the payload of MSC-EVs and yield procurement (Choi et al. 2019; Woo et al. 2020). For example, erythropoietin (100 IU/mL) supplementation of MSCs culture medium resulted in a significantly higher EVs production yield compared with the untreated control group; moreover, the secreted EVs were rich in anti-apoptotic miRNAs, i.e., miR-299, miR-499, miR-302, miRNA-200, and demonstrated greater therapeutic efficacy in experimental renal injury models both in vivo and in vitro (Wang et al. 2015). Lopatina et al. reported that MSCs stimulated by PDGF supplementation (20 ng/mL) increased the EV secretion rate and augmented proangiogenic capacity (Lopatina et al. 2014).

With ever-increasing information about EVs biogenesis mechanisms, key modulators and mechanisms associated with EVs secretion have been identified. Modification of specific molecular pathways in EVs biogenesis increase EV production (Phan et al. 2018). Recent studies have reported the activation of EVs biogenesis during membrane blebbing (P2X7 receptor, phospholipase D2), or multivesicular body fusion with the plasma membrane (Rab GTPase, SNARES) can increase the secretion of EVs, leading to an increased yield (Phan et al. 2018; Colombo et al. 2014; Qu and Dubyak 2009; Urbanelli et al. 2013; Rao et al. 2004; Hsu et al. 2010; Ostrowski et al. 2010; Laulagnier et al. 2004).

The homing ability of MSC-EVs towards ischemic and/or inflamed regions aids in delivering their payload more specifically to injury sites, contributing to maximizing therapeutic efficacies and minimizing systemic effects (Gudbergsson et al. 2019). These abilities of EVs (tissue tropism and cell-selective fusion) are attributed to the EVs membrane proteins determined by the phenotype of the parental cell (Peinado et al. 2012). However, recent findings revealed that after systemic injection, most of EVs are hardly free from the first-pass effect after accumulation in the liver, spleen, and lungs (Di Rocco et al. 2016). Therefore, the technologies to design and incorporate the targeting ligands of interest on the surface of EVs are valuable for enhancing the biodistribution and disease-targetability of EVs, eventually increasing their therapeutic efficacy (Kim et al. 2020; Man et al. 2020). One simple approach is

the direct insertion of hydrophobic molecules on the phospholipid bilayer of the EVs membrane for the hydrophobic attraction. Kim et al. reported the direct incorporation of aminoethyl anisamide-PEG onto the surface of EVs loaded with the anticancer drug paclitaxel to target lung cancer cells (Kim et al. 2018). The results showed enhanced accumulation of the functionalized EVs in lung cancer tissues after systemic injection, thereby improving the therapeutic outcomes. Kooijmans et al. used an alternative approach; fusogenic micelles conjugating EGF were successfully fused with EVs derived from platelets or Neuro2A cells without any configurational alterations in the size, morphology, and protein composition. The surface-modified EVs showed improved tumor-specificity and had longer stay in the blood circulation after systemic delivery in vivo (Kooijmans et al. 2016).

Shear stress at the physiological level contributes to the homeostasis of multiple tissues and organs in vivo, especially the tissues influenced by the presence of interstitial fluid flow or blood flow (Arora et al. 2020). Differentiation of MSCs into osteogenic, cardiogenic, chondrogenic, adipogenic, and even neurogenic lineages can be induced by varying different shear-stress conditions (0.01–2 Pa) (Arora et al. 2020). Shear stress can enhance the immune regulatory function of MSCs (Diaz et al. 2017). Likewise, the release of EVs from cells occurs inherently in response to shear stress at physiological or pathological levels in vivo. Therefore, as a bioinspired means to physically influence EVs production, controlled shear stress provided to the MSCs culture is considered an effective strategy to increase the yield of EV production and regulate its therapeutic composition (Piffoux et al. 2019). Hence, hollow fiber bioreactor technology that allows steady medium perfusion through massively bundled-up hollow microfibers provides great promise for MSCs-EVs production. This is because MSCs would constantly be under the controlled shear stress of a laminar flow condition. The maximum surface area would be available for cell seeding suitable for scaled-up culturing of MSCs (Colao et al. 2018). Furthermore, continuous medium perfusion can provide several vital practical benefits during MSC culture including: (1) the adequate mass transfer of oxygen, nutrients, and metabolites during the long-term culture period; (2) facilitate monitoring and controlling of culture parameters to maintain a well-defined MSC phenotype. These changes circumvent the unexpected alterations in the derivative EVs. Finally, retain the EVs product within a confined volume of the culture compartment during culture, which could yield more concentrated EVs in the conditioned medium (Cha et al. 2018a; Piffoux et al. 2019; Colao et al. 2018).

Besides mimicking the physiological features of EVs, the challenges in scaling-up the production of EVs are required be addressed for clinical applications. An automated cell culture platform based on the hollow fiber bioreactor technology would be one of the promising strategies for the scalable manufacturing and bioprocessing of therapeutically viable EVs products. This allows the large-scale production and prolonged culture of MSCs without phenotypic alterations and limited passage windows (Mendt et al. 2018). Concurrently, it provides MSCs with the controlled shear stress environment to modify and/or augment the therapeutic potential and yield of secreted EVs.

Many studies have reported that the intrinsic ability of MSCs to secrete a variety of therapeutic molecules is difficult to reproduce in vitro. This is because the natural 3D-interactions between cells and either ECMs or other neighboring cells are readily disrupted in conventional monolayer culture conditions, wherein the individual cells encounter limited 2D-borders (Placzek et al. 2009). Therefore, such conventional culture platforms are vastly problematic to reach the clinical-scale production of therapeutic EVs and demanding countless batches of MSCs with significant impact on labor, time, and cost. Given the lack of the physiologically relevant phenotype of MSCs resulting from the 2D-culture condition, the configuration of secreted EVs could be driven far from the natural compositions that MSC-EVs would have in the pathological conditions in vivo (Man et al. 2020). Accordingly, Nalamolu et al. reported that the treatment of EVs from 2D-cultured MSCs failed to improve the survival rate and adversely influenced recovery after stroke (Nalamolu et al. 2019). Such limitations of the monolayer culture have necessitated the development of 3D-culture platforms that closely mimic body's physiological microenvironment, which induces close cell-cell interaction and ensures improved cellular communication with highly cumulated signaling molecules (Cha et al. 2017). Numerous recent studies have reported that the formation of 3D MSC-aggregates can create a microenvironment akin to that in vivo wherein the phenotype and innate properties of MSCs are highly preserved (Bartosh et al. 2010; Frith et al. 2010). In a previous study, a novel 3D-culture platform using the microwell-array system was developed for the large-scale culture of 3D-MSCs spheroids. This 3D-system prevented cell loss, significant cost-saving without wasting expensive cell material, achieving highly reproducible and precisely controlled cell-size and cell number of the MSC spheroids (Cha et al. 2017). Furthermore, a simple and effective 3D-bioprocessing method has been developed using micro-well culture system for scalable production of therapeutically effective MSC-EVs. Moreover, 3D-culture increased the production of MSC-EVs enriched with angiogenic and neurotrophic factors (cytokines and miRNAs, respectively) approximately 100 folds more than the ones derived from 2D-culture system and minimized the uncertainty in cellular behaviors due to heterogeneous spheroid sizes (Cha et al. 2018b).

Stem cells detect ECM-derived mechanical cues that are conveyed through the cytoplasmic compartment and cause phenotypic changes as gene and/or protein expression profiles are inherently influenced (Engler et al. 2006). A previous study showed that varying stiffness of culture substrate influenced the secretory profiles of MSCs. For example, it significantly enhanced the secretion of pro-angiogenic factors (Abdeen et al. 2014). These data imply that different mechanical cues provided through the microenvironment can alter the fate of MSCs and their secretome profile. Our research group has investigated the effects of 3D-physical interactions between MSCs and culture matrix upon regulating the therapeutic compounds of MSC-EVs (unpublished data). In this study, MSCs encapsulated in GelMA hydrogels of varying stiffness from 9 to 21 kPa showed

differential gene expression profiles with the retention of innate characteristics of MSCs. The substantial upregulation of angiogenesis-related genes was observed besides higher mechanical properties. This data showed enhanced angiogenic capacity of the MSCs-derived EVs that was also confirmed by tubulogenic assay using human umbilical vein endothelial cells.

On the other hand, the interventions of 3D-biomaterial scaffolds can be supportive in regulating MSC-EVs. In a traumatic brain injury model, MSC-EVs cultured in the 3D-collagen scaffold exhibited a twofold increase in EVs secretion and functional recovery of traumatic brain injury due to neurogenesis and angiogenesis compared with MSC-EVs cultured in the 2D-culture system (Zhang et al. 2017). Native (including decellularized tissues), as well as synthetic polymer-based scaffolds, can be used as 3D-microenvironment to regulate cellular attachment, growth, migration, differentiation, and secretome profile (Phan et al. 2018). Based on the average EVs yield in previous studies, the estimated number of EVs secreted by 2D-cultured MSCs was approximately 500 EVs per cell, which was significantly higher (up to 100 folds) from the 3D-culture system (e.g., microwell culture or bioreactor system) (Kordelas et al. 2014; Cha et al. 2018b; Mendt et al. 2018).

Some researchers in the bioengineering field have focused their efforts to address the limitations in scalability and cost-effectiveness of the onerous purification steps and low production yield from the current EVs manufacturing protocols. The new concept of EVs bioprocessing is based on phospholipids' spontaneous self-assembly, the significant component of the membranous organelles and structures in a cell. The cells in defined culture conditions are subjected to physical processes, such as sonication and serial mechanical extrusions through filters of reducing pore sizes ranging from 1 μm to 100 nm and custom-made devices with hydrophilic microchannels or centrifuge modules, to disassemble them into nano-sized vesicles (Goh et al. 2017). The cell-derived nanovesicles (CDNs) may encapsulate endogenous cytosolic substances and therapeutic molecules of interest that are exogenously loaded during the plasma membrane self-assembly process. Moreover, molecular engineering techniques can modify cell membrane proteins to equip CDNs with customized ligands on their surfaces to specifically recognize the target disease sites (de Jong et al. 2019). Han et al. reported that a >300 times higher yield of CDNs than the amount of naturally secreted EVs could be obtained during MSC cultivation. Moreover, these CDNs possess attributes similar to those of the parental cells, MSCs (Han et al. 2019). These biofabricated CDNs have also demonstrated better skin wound healing potential in an experimental mouse model than the naturally secreted MSC-EVs. Most recently, successful preclinical outcomes with the use of CDNs were reported in regenerative medicine (Man et al. 2020). However, this EVs manufacturing approach has serious concerns regarding regulatory compliance, particularly regarding safety and quality control issues. These concerns emanate due to the high possibility of containing and shuttling undesirable factors such as genomic DNA compounds, unwanted metabolic molecules, and signaling molecules related to cell death.

Conclusions and Perspective

Unlike MSCs-based therapy, MSC-EVs therapy is still in the process of development. Currently, there are no Food and Drug Administration (FDA)-approved EVs-based therapeutic products. However, the use of MSC-EV therapy is rapidly expanding and could be a promising therapy for severe stroke patients as MSCs-based therapies have already been tested in preclinical and clinical trials. Compared with MSCs-based therapy, EVs-mediated therapy has unique advantages in terms of safety, biodistribution, stability, and off-the-shelf approaches for acute ischemic stroke. MSC-EVs therapy has advantages over conventional drugs or protein/RNA-delivery systems because MSC-EVs contain therapeutic payload with heterogeneous functions for recovery after stroke.

To date, the efficacy of MSC-EVs therapy has not been tested in stroke patients. An early phase clinical trial investigating the safety of allogeneic MSC-EVs in five patients with acute ischemic stroke is ongoing (clinicalTrial.gov. Identifier: NCT03384433). Our research group is also planning to conduct a phase 1/2a randomized trial on allogeneic Wharton's Jelly MSC-EV therapy (Stem Cell Extracellular Vesicle In Acute stroke trial). For successful clinical translation of EVs-based therapeutics, quality management and establishment of standard operating procedures for EV therapeutics as well as optimization of EV cargo and time/dose/mode of EV application for stroke patients are required.

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Mesenchymal Stem Cell Secretome: A Potential Biopharmaceutical Component to Regenerative Medicine

33

Bruna Araújo, Rita Caridade Silva, Sofia Domingues, António J. Salgado, and Fábio G. Teixeira

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B. Araújo · R. C. Silva · S. Domingues · A. J. Salgado
Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

F. G. Teixeira (✉)
Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

ICVS/3B's – PT Government Associate Laboratory, Braga/Guimaraes, Portugal
e-mail: fabioteixeira@med.uminho.pt

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Abstract

Over the last decade, the use of stem cells has remarkably been proposed as a regenerative tool, and within it, mesenchymal stem cells (MSCs) have emerged as a promising therapeutic option. As a consequence, they currently represent an effective tool in the treatment of several diseases due to their tissue-protective and tissue-reparative properties. Based on these MSCs' regenerative potentialities are the secretion and release of trophic molecules and vesicles, nowadays known as stem cell secretome. Notably, MSCs' secretome itself is starting to be considered a potential active pharmaceutical component, in which its vesicular portion has been revealing promising characteristics to be used as a drug delivery system, thereby opening an opportune window to the specific release of drugs, peptides, or specific agents to targeted damaged areas. Therefore, the use of MSCs' secretome as a whole or its components per se has demonstrated remarkable advantages over cell transplantation procedures, with no adverse effects, thereby indicating that it can be used as a source of bioactive agents that can be efficiently stored and transported as a ready-to-use biocomponent. Thus, on the scope of the present chapter, we intend to provide an overview of the application of MSCs' secretome as a therapeutic strategy to the regenerative medicine field.

Keywords

Mesenchymal stem cells · Reparative properties · Secretome · Therapeutic strategy · Regenerative medicine

Abbreviations

AD	Alzheimer's disease
AKI	Acute kidney injury
ALI	Acute lung injury
AMPK	5' Adenosine monophosphate-activated protein kinase
Ang-1	Angiopoietin-1
ARDS	Acute respiratory distress syndrome
ASCs	Adipose-derived stem cells
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BSCB	Blood-spinal cord barrier
circRNAs	Circular RNAs
CKD	Chronic kidney disease
CM	Conditioned medium
CNS	Central nervous system
CSCs	Cardiac stem cells
CXCR4	C-X-C chemokine receptor type 4
ECM	Extracellular matrix

ECs	Endothelial cells
EG-VEGF	Endocrine gland-derived vascular endothelial growth factor
EVs	Extracellular vesicles
FGF-BP	Fibroblast growth factor-binding protein 1
FPHL	Female pattern hair loss
GAP-43	Growth-associated protein 43
GvHD	Graft-versus-host disease
hAMSCs	Human adipose tissue-derived MSCs
HD	Huntington's disease
HGF	Hepatocyte growth factor
hMSCs	Human mesenchymal stem cells
IGF	Insulin-like growth factor
IL	Interleukin
IS	Ischemic stroke
ISCT	International Society for Cellular Therapy
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factor
lncRNAs	Long noncoding RNAs
MAPK	Mitogen-activated protein kinase
mHtt	mutant Huntingtin
miRNAs	microRNAs
MMP-13	Matrix metallopeptidase 13
MRCTs	Massive rotator cuff tears
mRNA	Protein-coding messenger
MS	Multiple sclerosis
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
MVBs	Membrane of multivesicular bodies
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
NO	Nitric oxide
NT-3	Neurotrophin-3
OA	Osteoarthritis
PD	Parkinson’s disease
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinases
PK1	Pyruvate kinase 1
RNAseq	RNA sequencing
SCI	Spinal cord injury
SDF-1	Stromal cell-derived factor-1
TBI	Traumatic brain injury
TGF-ß1	Transforming growth factor ß1
TIMP3	Tissue inhibitor of matrix metalloproteinase-3

TSG	Tumor necrosis factor-stimulated gene
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand factor

Introduction

In normal conditions, the organs and tissues of our body have limited regenerative capacities, which indicate that in case of failure of those systems, life-threatening conditions can arise. Moreover, many diseases result from the interface between complex mechanisms, including cellular impairments and tissue or organ dysfunctions. Among them, difficulties in treatment are prevalent since classic strategies display an inability to efficiently stimulate vital mechanisms of tissue regeneration, repair, and renewing and to promote the reversion of cell loss (Hoda Elkhenany et al. 2020).

Over the past decades, the field of stem cell-based research has gained particular interest under the scope of novel regenerative/repairing therapeutic opportunities, as it appears to target several disorders and public health issues for which current medical and surgical solutions are insufficient. From a spectrum of several stem cell populations, mesenchymal stem cells (MSCs) have become a pivotal population in developing promising therapies involving the maintenance and repair of adult tissues and organs.

MSCs' Roots

During the 1960s, it was with the trailblazing work of Friedenstein and colleagues that MSCs were first isolated from rodent bone marrow (BM) and characterized as a rare population of adherent clonal non-hematopoietic precursors, capable of differentiating into mesodermal-derived cell types (Caplan 1991; Friedenstein et al. 1968). These pioneer observations sparked a significant degree of curiosity and led to the elaboration of heterogeneous procedures in isolation and cultivation of them among laboratories. For this reason, the International Society for Cellular Therapy (ISCT) established, in 2006, minimal criteria to classify a cell population as MSCs, namely, the (1) plastic adherence when maintained under standard culture conditions, (2) phenotypic expression of specific surface antigens (CD73, CD90, CD105) and concomitant absence of hematopoietic markers (CD14, CD34, CD45, and human leucocyte antigen-DR), and (3) tri-lineage mesenchymal differentiation toward osteoblasts, adipocytes, and chondroblasts in vitro (Dominici et al. 2006). However, ever since, numerous reports have confirmed that MSCs are not only present in the bone marrow and have now been isolated from a variety of non-marrow tissues using different protocols, including the placenta, skeletal muscle, adipose tissue, umbilical cord, Wharton's jelly, peripheral blood, lung, liver, dermal tissue, and even brain (Chamberlain et al. 2007; Venkataramana et al. 2010; Phinney 2007; Praveen Kumar et al. 2019). Notably, various studies have revealed that, despite their uniformly marked

profiles and similar cellular phenotypes, MSCs derived from different tissues exhibit a differentiation potential broader than initially thought (Phinney 2007; Harrell et al. 2019). These data indicate that under specific niches, conditions, and cellular microenvironments, these cells can "transdifferentiate" at different rates due to plasticity and modify their biological and functional characteristics accordingly (Praveen Kumar et al. 2019; Squillaro et al. 2016; Paul and Anisimov 2013). Moreover, epigenetic findings in MSCs from distinctive tissues determined the existence of substantial differences in gene expression patterns, transcriptome/proteome, and functionality that were associated merely with the tissue source (Phinney 2007; Le Blanc and Davies 2018). Thereby, when applying different protocols for their isolation and cell culture expansion, MSCs could generate cells of neuroectodermal and endodermal origin, including neuron-like cells, hepatocytes, pancreatic islet-like cells, cardiomyocytes, and alveolar and gut epithelial cells (Harrell et al. 2019; Squillaro et al. 2016; Teixeira et al. 2013; Trohatou and Roubelakis 2017; Chen et al. 2004). Additionally, besides the heterogeneity observed among MSCs from different sources, there are also variances after acquiring them from individual donors (Andrzejewska et al. 2019). Overall, these assumptions support the idea that MSC benefits organ and tissue repair due to their multipotency to generate cells of the aimed tissue and substitute damaged resident cells (Squillaro et al. 2016).

MSCs' Biological Properties

MSCs were primarily used for musculoskeletal regeneration and wound healing. Nonetheless, and acknowledging their benefits afar from tissue repair, they have been primarily used in numerous experimental, pre-clinical, and clinical models, involving organ transplantation, cancer, rheumatic diseases, autoimmune diseases, inflammatory disorders, spinal cord injuries, acute ischemic stroke, diabetes, neurodegenerative disorders, microbial infections, myocardial infarction, and so forth (Ferreira et al. 2018; Serra et al. 2018; Bai et al. 2016; Hu and Li 2018). Given that, a comprehensive study of the molecular and biological properties that define the MSCs is critical (Bai et al. 2016).

One of the most common features of MSCs is, as previously mentioned, their undifferentiated self-renewal ability along with multi-lineage differentiation potential that significantly influences tissue homeostasis (Eleuteri and Fierabracci 2019; Mendes-Pinheiro et al. 2020). In contrast, these cells present further multifunctional characteristics, which embrace not only immunomodulation and homing capability but also pro-angiogenic, antioxidant, antimicrobial, neuroprotective, anti-tumorigenic, anti-apoptotic, and chemoattractive effects (Squillaro et al. 2016; Bai et al. 2016; Murphy et al. 2013; Spees et al. 2016). Given the immunomodulatory functions of MSCs, it has been reported that they can switch their profile from an innate to acquired immune response or vice versa, either by endorsing pro-inflammatory events if the level of inflammatory cytokines is low or by negatively regulating the immune response upon an inflammation, often relying on the

context, local microenvironment, and disease status (Murphy et al. 2013; Harrell et al. 2019; Andrzejewska et al. 2019; Song et al. 2020). Likewise, MSCs can interact with immune cells via cell-to-cell contact and interfere with their proliferation, activation, and function. Of note, peripheral blood mononuclear cells (monocytes), neutrophils, B and T (including regulatory T cells) lymphocytes, natural killer cells, macrophages, and dendritic cells are all possible targets of the interference of these cells (Harrell et al. 2019; Song et al. 2020; Gao et al. 2016). Connecting to this effect, the homing capability of MSCs centers its attention on their migratory behavior and capacity to navigate and reach damaged tissue as feedback to a combination of cytokines (Nitzsche et al. 2017). For instance, considering its potential of tumor inhibition and tropism, it is also worth mentioning that MSCs can be loaded with chemotherapeutic agents and successfully deliver their payload in site-directed manner to the tumor sites (Kwon et al. 2019). Nevertheless, the majority of the previously mentioned potentialities are the outcome of the paracrine activity-based mechanisms of these cells. Indeed, it has been considered that the array of bioactive factors they secrete in response to the local environment constitutes the mechanism by which MSCs assist many of their beneficial effects.

The Pitfalls of MSCs

Notwithstanding the promising results obtained in the clinical trials, MSC-based therapies are not considered a standard of care at the clinic, facing several obstacles to their applicability (Squillaro et al. 2016; Zaher et al. 2014). Firstly, there is an obvious lack of standardized procedures regulating cell culture, ex vivo expansion, cryopreservation, and differentiation, which affects MSCs' properties and, as a consequence, leads to stemness attenuation and replicative senescence (Praveen Kumar et al. 2019; Squillaro et al. 2016; Kwon et al. 2019; Kandoi et al. 2018). Also accompanying this issue is the large variability in cell quality, due to the usage of different donors and their tissues, known as donor heterogeneity (Squillaro et al. 2016; Teixeira and Salgado 2020; Gao et al. 2016). Equally, regarding transplantation, a significantly high number of cells are required to have a notable effect (Teixeira and Salgado 2020; Vizoso et al. 2017). Posterior to this procedure, not only do MSCs exhibit a low survival rate (Hu and Li 2018) but also do not commonly become a part of the injured site, a detail previously revealed by cell tracking analysis (Teixeira et al. 2013). Furthermore, hurdles in defining a correct therapeutic rationale, including determining optimal dosage type, administration frequency, mode of infusion, and effective delivery route, are likewise points to consider when selecting these cells for transplantation.

Given the drawbacks mentioned above, more recently, the use of MSCs per se has been reconsidered, and in its place, a direct application of their secreted trophic factors is being evaluated as part of fast-emerging cell-free therapy approach (Gnecchi et al. 2005; Haider and Aslam 2018), since those might be the essential foundations required to create effective cellular strategies.

Secretome Derived from MSCs as Cell-Free-Based Therapeutic Strategy

The plethora of protective bioactive factors secreted by MSCs is known as stem cell secretome or conditioned medium (CM). These paracrine factors released by a cell, tissue, or organism into the extracellular space can promote their repair and regeneration (Mendes-Pinheiro et al. 2020). Secreted molecules play a valuable contribution in crosstalk communication between cells and the surrounding tissues. Therefore, the MSCs' secretome-based therapies are considered as an appealing proposal to be used in several domains of regenerative medicine (Haider and Aziz 2017). Like MSCs, the secretome profile depends on the MSCs' source and culture conditions used to expand the cells (Yin et al. 2019). The factors and their concentration used may diverge depending on cellular and preparation parameters and by intrinsic and extrinsic environment conditions (Kehl et al. 2019; Bundgaard et al. 2020). Owing to that dissimilarities, the secretome composition may be manipulated to obtain the an optimal bioproduct profile for a specific therapeutic application and to identify the regenerative mechanisms of distinct tissue types/origins (Chang et al. 2021).

Composition and Characterization of MSCs' Secretome

The secretome composition is determined by the conditioned medium after MSCs culture in vitro. It can be divided into two distinct fractions – the soluble fraction (essentially proteins and soluble factors, such as cytokines) and the vesicular fraction (Pinho et al. 2020) (Fig. 1). A plethora of protective bioactive factors classified as growth factors, chemokines, cytokines, microRNA, hormones, free nucleic acids, lipid mediators, extracellular vesicles (EVs), and other small molecular weight signal cues constitute the secretome composition, creating a microenvironment suitable for cellular repair and regeneration (Ranganath et al. 2012; Beer et al. 2017; Hu et al. 2020). Those molecules influence the crosstalk communications between cells and the surrounding tissue to stimulate the recruitment, proliferation, and differentiation of the endogenous cells. Regarding EVs, these are secreted by the MSCs transporting active molecules and genetic information to target cells. They contain growth factors, cytokines, miRNA, and mRNA that trigger several biological responses in the target area (Rani et al. 2015). More recently, it has been accepted that when used alone, the EVs may provide a similar or enhanced therapeutic advantage compared to the cells secreting the secretome (Phelps et al. 2018).

The Soluble Fraction: Cytokines and Growth Factors

Regarding the soluble fraction of MSCs' secretome, the most physiologically relevant biomolecules secreted include basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF), which are involved in immunomodulation, cell migration, development, and regulating apoptosis; vascular endothelial growth factor (VEGF) which is a critical regulator of angiogenesis, immunomodulation,

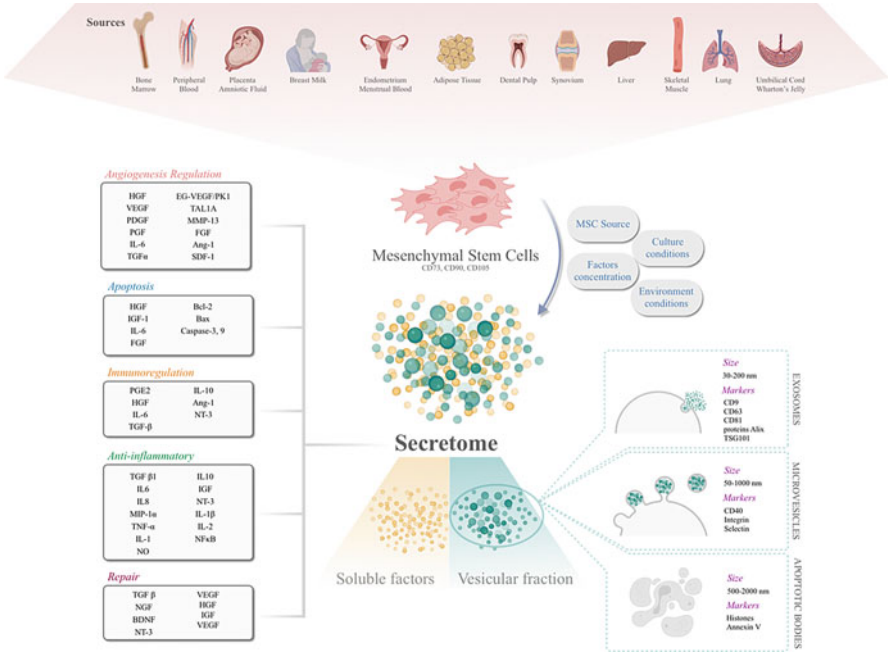

Fig. 1 The possible sources and composition of MSCs' secretome. When exposed to different conditions and in response to pathological processes, MSCs secrete a plethora of factors for the repair and regeneration of the host tissue. The therapeutic benefits are determined by the secretome composition. The secretome is divided into soluble fraction (cytokines and factors) and vesicular fraction (extracellular vesicles)

and cell survival; transforming growth factor ß1 (TGF-ß1) which targets immunomodulation, cell growth, proliferation, and differentiation; tumor necrosis factor-stimulated gene (TSG-)6, prostaglandin E2 (PGE2), and galectins 1 and 9 that are also associated with immunomodulation and anti-inflammatory functions (Noone et al. 2013; Madrigal et al. 2014; Gieseke et al. 2013); and finally insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), and interleukin 6 (IL-6) that are mainly angiogenic and immunomodulatory (Martín-Martín et al. 2019; Phelps et al. 2018). Genetic modification of MSCs with IGF-1 or the concomitant overexpression of pro-survival and pro-angiogenic factors, i.e., Akt and angiopoietin-1, significantly enhanced the paracrine activity of the cells and supported angiomyogenic repair of the infarcted heart in experimental animal model (Haider et al. 2008; Jiang et al. 2006).

The Vesicular Fraction: Extracellular Vesicles

Extracellular vesicles are important for carrying components from donor cells to recipient cells serving as intercellular mediators of communication (Marote et al. 2016). They are phospholipid membrane-bound particles that contain biological material (DNA, RNA), bioactive lipids, and proteins. However, EVs' composition

depends on the MSCs' source; the donor-related factors such as age, health condition, etc.; genetic and epigenetic memory of the cells; the pathological state of the cells; etc. (Raposo and Stoorvogel 2013).

The EV is a general term for various vesicles secreted by MSCs and include exosomes, microvesicles, and apoptotic bodies (Raposo and Stoorvogel 2013). Their characterization is based on the size, origin, and markers. For instance, exosomes (30–200 nm) originate from the internal budding of the membrane of multivesicular bodies (MVBs), a subset of endosomes that contains membrane-bound intraluminal vesicles. Afterward, it is released into the extracellular environment, subsequent to fusion with the plasma membrane. Therefore, their small size simplifies the transfer through blood and to other biological fluids. Exosomes are classified by CD9, CD63, and CD81, proteins Alix, and TSG101-positive expression. In terms of characterization, microvesicles (50–1000 nm) sprout directly from the plasma membrane and are identified by CD40 marker, integrin, and selectin-positive expression. Finally, apoptotic bodies (500–2000 nm) include fragments of dead or dying cells and are characterized by the presence of histones and annexin V-positive staining (Phelps et al. 2018) (Fig. 1).

Thus, considering the paracrine signaling as the primary therapeutic mechanism of MSCs, many promising in vivo studies have reported the use of MSCs' secretome in toto or in the form of fractionated MSC-derived EVs as a promising therapeutic approach. Recent studies have evidenced that exosomes may be primarily responsible for the therapeutic effects of the MSCs' secretome (Shao et al. 2017; Nakamura et al. 2015; Furuta et al. 2016). However, the benefits of EVs have been only been attributed to the cytokines and growth factors but also to the RNAs and miRNAs, which play an essential role in gene expression regulation and the surrounding cell/tissue's microenvironment (Shao et al. 2017).

The Vesicular Fraction: Coding and Non-coding RNAs

In addition to the protein fraction of the secretome, MSCs also secrete protein-coding messenger (mRNA) and non-coding RNAs such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) via their EVs, which can regulate the various cellular functions and activities, i.e., cell cycle, metabolism, migration, inflammation, and angiogenesis. The mRNA component can be transported and delivered to the recipient cells to cause changes in their protein or gene expression profile (Phelps et al. 2018). The miRNAs are important constituents of the cell secretome and contribute essentially in providing the therapeutic effects of the secretome in terms of repair and regeneration of the injured tissue. They are associated with the expression of proteins related with apoptosis and cell survival, stem cell differentiation, hematopoiesis and vascular development (miR-23), insulin secretion, cell growth (miR-125b), angiogenesis (miR-29), and immune response (Zhang et al. 2017; Wang et al. 2017; Ferguson et al. 2018; Tsukita et al. 2017). On the other hand, the lncRNAs are involved in chromatin organization, gene transcription, mRNA turnover, protein translation, assembly of macromolecular complexes, etc. (Ng et al. 2012; Gezer et al. 2014; Kim et al. 2017). Also, the circRNAs have shown the capacity to take advantage of miRNAs and control their

function as regulators of mRNA stability and/or translation (Kim et al. 2017). Still, in EVs, they could constitute a potential biomarker in the disease processes as well as therapeutic targets as they play a pivotal role in diverse cellular processes and cell fate determination (Kim et al. 2017; Bao et al. 2016).

Mechanisms of Action and Principal Effects

Cell secretome-based therapy has some distinct advantages over the cell-based therapy approach, rendering their use a promising therapeutic strategy. Autocrine or paracrine effects of the MSCs rather than direct engraftment and tissue differentiation play an essential role in tissue repair. Due to the excellent pro-proliferative and anti-apoptotic effects and given their potent trophic properties, MSCs' secretome has emerged as a prospective therapeutic tool for numerous clinical applications. Indeed, MSCs' secretome targets some major biochemical processes, providing anti-inflammatory, pro-angiogenic, immunomodulatory, anti-fibrotic, and anti-apoptotic effects, besides promoting cell proliferation and supporting migration and homing-in and retention of the inherent stem/progenitor cells to the site of injury for participation in the ongoing repair process (Chang et al. 2021; Hu et al. 2020; Xia et al. 2019).

Pre-clinical Approaches of Mesenchymal Stem Cell Secretome

The presence of trophic factors and exosomes offered by MSCs' secretome has expanded its utility as a cell-free therapy. In recent years, a large number of pre-clinical studies have been reported that show that the administration – either systemic or local – of the secretome from MSCs derived from different tissue sources possess distinct therapeutic benefits when applied for the treatment of different diseases, e.g., inflammatory and degenerative diseases of hepatobiliary, respiratory, skeletal, gastrointestinal, cardiovascular, and nervous systems. Treatment with MSC-derived conditioned medium successfully reverted the characteristic damaging phenotypes (Vilaça-Faria et al. 2019) (Fig. 2). Herein, we will briefly dissect the distinctive in vitro and in vivo experimental approaches in animal models wherein MSCs' secretome was applied.

Wound Healing and Cartilage Repair and Regeneration

Under different experimental conditions, MSCs' secretome has shown its proficiency to promote wound closure and healing in diverse types of lesions. A study by Heo et al. demonstrated that upon administration of human adipose tissue-derived MSCs' (hAMSCs') secretome into an experimental rat excisional wound model, there was a significant angiogenic response evidenced by increased vascular density,

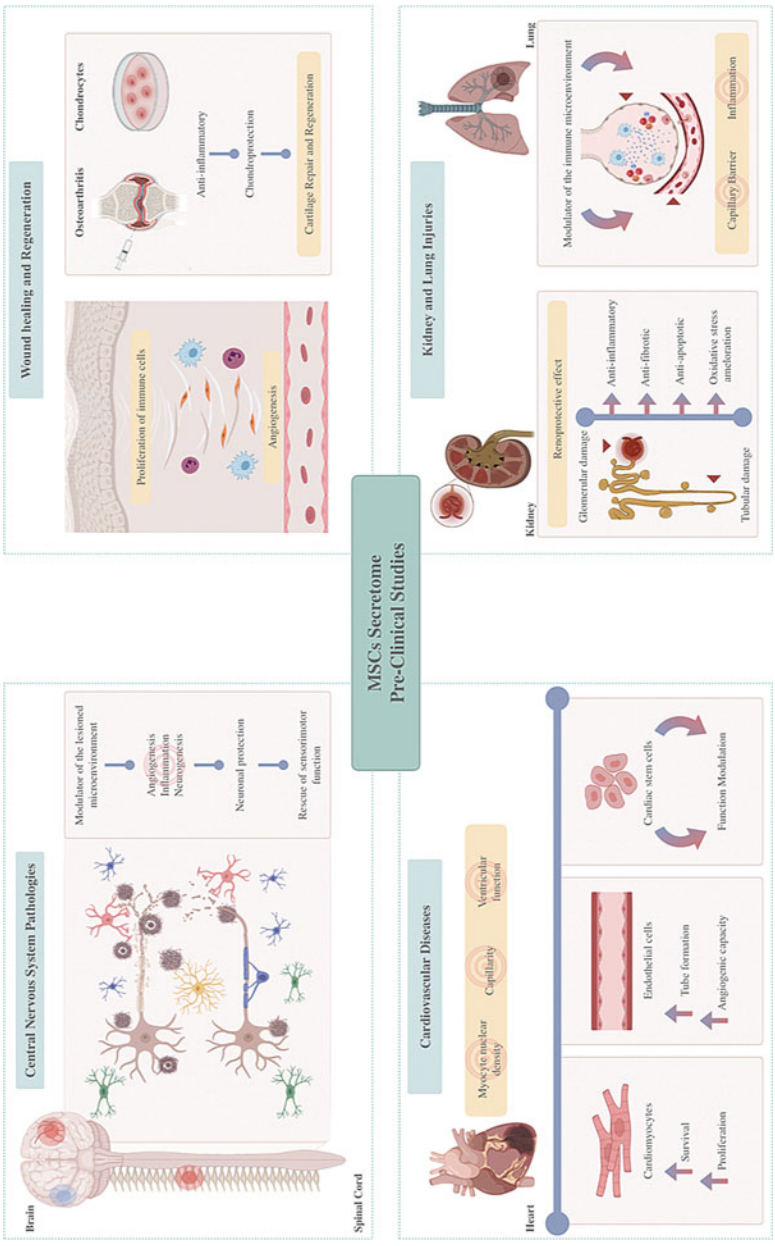

Fig. 2 Summary of the encouraging diverse therapeutic benefits of MSC-derived secretome in distinct disorders and lesions. Pre-clinical studies suggest that the secretome and its components have the capability to enable the regeneration of the injured brain, spinal cord, heart, bone, kidney, and lungs by immunomodulating the injury microenvironment and protecting the tissues, targeting different cell types and mechanisms

wound closure, and proliferation/infiltration of immune cells, mostly in response to IL-6 and IL-8 (Heo et al. 2011; Makridakis et al. 2013).

On the same line of thoughts, another report in an experimental rat model of dry eyes revealed that following injury, the secretome was able to enhance epithelial regeneration and reduce mRNA expression of corneal macrophage inflammatory cytokines, such as protein-1α (MIP-1α) and TNF-α (Vizoso et al. 2017; Bermudez et al. 2015). Moreover, when exploring healing strategies for massive rotator cuff tears' (MRCTs') lesions in an experimental rat model, our research group has reported that treatment with hMSC-derived secretome successfully reduced the fatty degeneration and atrophy of the muscles. Molecular studies revealed that hMSC-derived treatment increased pigment epithelium-derived factor (PEDF) and follistatin expression (Sevivas et al. 2017). Analogously, SCs also have the capacity to accelerate the formation of bony scars. Using umbilical cord-derived secretome, several studies have shown its capability to promote skin wound healing with the formation of fewer scars, through the stimulation of macrophage and endothelial migration homing-in and retention at the site of the injury, myofibroblast differentiation, and expression of extracellular matrix (ECM) genes (Li et al. 2017; Jackson et al. 2012; Baez-Jurado et al. 2019).

Similarly, published data suggest using MSCs' secretome for osteoarthritis (OA), wherein chondrocytes exhibit a damaging role in the cartilage degeneration that contributes to the progression of the disease. Using in vitro experimental models, researchers have found that treatment with MSC-derived CM or its derived extracellular vesicles (exosomes) decreased the inflammatory phenotype of OA chondrocytes by downregulating the expression of inflammatory cytokines (TNF-α, IL-1, IL-6, nitric oxide (NO)) and enhancing the production of immunosuppressive IL-10. These cellular and molecular changes contributed toward the anti-inflammatory and chondroprotective effects of the CM treatment (Tofiño-Vian et al. 2018; Chen et al. 2018). These positive consequences of the secretome in OA were similarly confirmed in vivo. Using experimental animal models of OA, MSC-sourced secretome administration enhanced the formation of new tissue and increases type II collagen synthesis (Zhang et al. 2016a). Similarly, treatment with MSC-derived extracellular vesicles stimulated endogenous cartilage repair and regeneration, primarily associated with specific exosomal miRNAs to re-establish to basal level (homeostasis) of metabolism in the proliferating chondrocytes (Liu et al. 2018a, b; Sun et al. 2019; Toh et al. 2017; Harrell et al. 2019).

Additionally, it has also been observed that cell-derived secretome showed the ability to:

1. Reduce hypertrophy and de-differentiation of chondrocytes in culture via the secretion of hepatocyte growth factor (HGF), which may be noteworthy for other osteoarticular disorders since it could offer chondroprotection (Maumus et al. 2013)
2. Induce bone regeneration (as observed in rabbit's mandibles) after surgical lesions (Linero and Chaparro 2014)

Cardiovascular Diseases

Although there has been an evolution in treatment options, cardiovascular diseases continue to be major causes of morbidity and mortality globally (Squillaro et al. 2016). Hence, in recent years, MSC-sourced secretome has been used for cardiac regeneration as the fast-emerging cell-free therapy approach (Lei and Haider 2017). Experimental animal studies have shown that secretome treatment per se improved left ventricular function, heightened myocyte nuclear density and neovascularization, and reduced apoptosis/fibrosis in the ischemic heart (Baez-Jurado et al. 2019; Shabbir et al. 2009; Dai et al. 2007). Nonetheless, the cardioprotective effects of secretome-based treatment have been attributed to the MSC-derived EVs that affect the cellular targets, i.e., cardiomyocytes, endothelial cells (ECs), and cardiac stem cells (CSCs) (Suzuki et al. 2016; Zhang et al. 2016b; Silva et al. 2017). Actually, and by using experimental animal models of cardiovascular dysfunction, several studies showed that MSC-derived exosomes could notably reduce the infarct size and enhance global cardiac function (Harrell et al. 2019; Suzuki et al. 2016; Silva et al. 2017; Lai et al. 2010). Arslan et al. in one of such cases demonstrated that exosome-treated animals showed suggestive conservation of the left ventricular geometry and the contractile performance through the re-establishment of myocardial bioenergetics, a reduction in oxidative stress, and activation of the PI3K/Akt pro-survival signaling pathway (Harrell et al. 2019; Arslan et al. 2013).

Explicitly considering the cardiomyocytes, various studies have shown that the administration of MSC-derived exosomes increased the survival and proliferation of cardiomyocytes by inhibiting apoptotic signaling pathways and stimulation of autophagy (Harrell et al. 2019; Ju et al. 2018). For example, Liu et al. reported that MSC-derived exosomes promoted the autophagy process by upregulating the AMPK/mTOR and Akt/mTOR signaling pathways (Harrell et al. 2019; Liu et al. 2017). Similarly, Cui et al. showed that the protection of cardiomyocytes against apoptosis was due to an increased expression of anti-apoptotic protein, Bcl-2, and concomitant downregulation of pro-apoptotic Bax protein expression. In addition, they also observed suppression of caspase-3 activity in response to exosome treatment (Harrell et al. 2019; Cui et al. 2017). Also, treatment with exosomes successfully supported microvascular regeneration (Harrell et al. 2019; Gong et al. 2019; Ma et al. 2018). Supporting these findings, the delivery of specific microRNAs presented the capacity to modulate CSCs, by either stimulating their proliferation and migration or increasing their capacity of self-renewal (Harrell et al. 2019; Zhang et al. 2016b).

Furthermore, the cardiac benefits of MSC-derived exosomes were associated with the inhibition of the inflammatory reaction. Indeed, extracellular vesicles contributed to the regulation of immune cell function, controlling macrophages' polarization, and diminishing the influx of inflammatory cells into treated hearts (Harrell et al. 2019; Silva et al. 2017; Sun et al. 2018). This is accomplished by delivering exosomal payload of miRNAs to the heart, the composition of which may be altered by exogenous manipulation of the cells of their origin (Haider and Aramini 2020).

Kidney and Lung Injuries

Some of the other models in which MSCs' secretome has displayed ameliorative benefits include chronic kidney disease (CKD) and acute kidney injury (AKI). Regarding CKD, the administration of MSC-derived secretome promoted marked (reno)protective effects, which were revealed by a decrease in glomerular damage and hypertension besides improved glomerular endothelial regeneration and genome integrity preservation via active DNA repair (Van Koppen et al. 2012). Likewise, the same research group observed in vitro that treatment with secretome improved endothelial cell migration and angiogenesis, followed by a reduction in tubular inflammation and fibrosis, which indicated an anti-inflammatory and anti-fibrotic effect. These data were consistent with the previously published studies in other experimental disease models (Van Koppen et al. 2012). For example, Togel et al. attributed the renoprotective effects of the secretome to various growth factors, including VEGF, HGF, and insulin-like growth factor (IGF) (Tögel et al. 2009). Nevertheless, reports in AKI experimental models have encouraged the use of MSC-derived microvesicles for kidney protection (Gatti et al. 2011; Zhou et al. 2013b; Bruno et al. 2012). In fact, Gatti et al. showed that one-time administration of microvesicles suppressed apoptosis and boosted tubular epithelial cell proliferation, which considerably attenuated renal function impairment (Gatti et al. 2011). Some other research groups have confirmed that the pro-survival effects of secretome were mostly ascribed not only to anti-apoptotic effects but also to an amelioration of oxidative stress (Zhou et al. 2013b, Bruno et al. 2012).

Parallel to this, lungs' MSC-derived exosomes have been reported in experimental animal models of acute lung injury (ALI), acute respiratory distress syndrome (ARDS), asthma, and idiopathic pulmonary fibrosis (Squillaro et al. 2016; Harrell et al. 2019). Considering ALI and ARDS, there is an obvious lack of therapeutic options to prevent/treat injury and/or promote lung repair. For example, in vivo data have shown that secretome administration successfully attenuated lung inflammation and activation of macrophage to an M2 "healer" phenotype, partially influenced by the presence of IGF-I (Ionescu et al. 2012). In another report, researchers found out that ALI treatment with MSC-derived vesicles mostly targeted the immunomodulatory properties of angiopoietin-1 (Ang-1) that successfully led to a reduction in inflammation and vascular stabilization (Tang et al. 2017). In experimental ALI models, treatment with microvesicles significantly reduced pulmonary edema and decreased the influx of both inflammatory cells, proteins, and bacteria, in a keratinocyte growth factor (KGF)-dependent manner (Zhu et al. 2014; Monsel et al. 2015), thereby opening new therapeutic opportunities for this kind of disorders.

Central Nervous System Pathologies

Cerebral homeostasis is preserved through complex and cohesive interactions between distinctive cell types, comprising neurons and glial cells. In the central nervous system (CNS) pathologies, the physiology of these cells gets highly changed, leading to

impairments in their function and altering the protection and balance in the nervous tissues. Usually, brain disorders display typical biological hallmarks such as an intensification of reactive oxygen/nitrogen species production, protein aggregation and denaturation, secretion of apoptotic factors, metabolic alterations, mitochondrial dysfunction, and, as an outcome, huge percentages of neuronal and glial cell death populations. Since CNS lacks the capability of self-repair, or at least limited self-repair, it is challenging to design treatments that could prevent the progression of injury and concomitantly halt the cognitive and motor/non-motor functional decline during pathologies, such as traumatic brain injury (TBI), ischemic stroke, spinal cord injury, and neurodegenerative diseases (Teixeira et al. 2013; Baez-Jurado et al. 2019; Mendes-Pinheiro et al. 2020). In this regard, MSCs' secretome has shown its potential as a therapeutic tool to brain tissue recovery and repair, owing to its capability to trigger and/or modulate endogenous neuro-restorative processes, such as neurogenesis, angiogenesis, and inflammation (Teixeira et al. 2013; Makridakis et al. 2013; Pinho et al. 2020; Mendes-Pinheiro et al. 2020).

Traumatic Brain Injury (TBI)

TBI is a global health challenge that arises through external mechanical forces triggering injury and disrupting normal brain function (Pinho et al. 2020). Two main physiopathological phases are generally involved in this disorder, englobing firstly the mechanical impairments (disruption of the blood-brain barrier and disseminating axonal damage) and, secondly, chronic inflammation due to the excessive production of pro-inflammatory cytokines, mitochondrial breakdown followed by oxidative stress, and excitotoxicity (exaggerated glutamate levels) (Baez-Jurado et al. 2019; Muhammad 2019; Mendes-Pinheiro et al. 2020). As a consequence of these molecular events, patients develop physical, cognitive, and emotional deficits. Therefore, the use of MSCs' secretome is being considered as a promising therapeutic opportunity to treat TBI, demonstrating potential modulatory effects in the lesioned microenvironment. Indeed, during the acute phase of the injury, treatment of TBI with secretome containing neurotrophic factors (NGF, BDNF, NT-3) supported rats' neurological repair and reduced apoptosis (Kim et al. 2010). Moreover, the same authors have shown that TBI-treated rats showed improvement in motor function and cognitive performance, essentially due to an increase in VEGF and HGF expression, which was correlated with neurogenesis in the damaged tissue areas (Chang et al. 2013; Chuang et al. 2012). In an experimental acute TBI model, MSC-secreted soluble factors were significantly modified the expression of pro- and anti-inflammatory cytokines and modulated the serum levels of chemokines due to their immunomodulatory properties (Galindo et al. 2011). Similarly, Pischiutta et al. concluded that upon amniotic mesenchymal stromal cell-secreted metabolite administration, brain slices of TBI mice developed protective effects, including the promotion of M2 microglia polarization and neuronal rescue (Pischiutta et al. 2016). Additionally, in a study using a new in vitro experimental model of traumatic brain-like injury, researchers have found that hMSC-derived secretome could

preserve cell morphology and their polarity index to increase wound closure, migration, and proliferation and control the oxidative stress by decreasing superoxide production (Torrente et al. 2014). Comparably, using adipose-derived stem cells' (ASCs') secretome, Tajiri et al. suggested that the secretome could support/boost endogenous repair mechanisms by improving motor/cognitive behavior but also by avoiding cortical and hippocampal damage. More importantly, they found that the major players involved in this efficacy were the lncRNAs (Tajiri et al. 2014). More recently, in an SH-SY5Y model of TBI, apart from diminishing neuronal cell death, the therapeutic application of ASC-derived secretome significantly increased mitochondrial function and reduced inflammatory cytokine IL-1β (Kappy et al. 2018). Consistent with the published data, RNA sequencing (RNAseq) revealed that hMSCs' secretome administration normalized the expression of 49% of the genes disrupted by TBI, especially in specific pathways that were involved in immune cell signaling and infiltration, energy metabolism, receptor-mediated cell signaling, and neuronal plasticity and remodeling (Darkazalli et al. 2017). In an attempt to understand the underlying molecular mechanism, different groups showed that proteins such as Wnt3a and tissue inhibitor of matrix metalloproteinase-3 (TIMP3) had a role in the promotion of cerebral endothelial adherent junction integrity, neuroprotection, and neurocognitive function (Zhao et al. 2016; Gibb et al. 2015). Having all this in mind and following in the same direction, the use of the extracellular vesicles secreted by MSCs has gained particular interest in TBI. Indeed, treatment with MSC-derived EVs is described as a potential promoter of neurological recovery through either of the following mechanisms:

(i) Modulation of inflammation-related pathways, in which they shifted microglia polarization and prevented reactive astrogliosis
(ii) Inhibition of pro-apoptotic proteins and oppositely an increase in the expression of the anti-apoptotic ones
(iii) Restoration of myelination deficits and white matter microstructure
(iv) A significant increase in the neurogenesis of endothelial cells, neuroblasts, and mature neurons in the lesion site
(v) Rescue of the sensorimotor function (specially learning and motor performance), opening new routes for future clinical translation (Williams et al. 2019; Cantinieaux et al. 2013; Thomi et al. 2019)

Spinal Cord Injury (SCI) and Ischemic Stroke (IS)

Numerous studies have indicated that in addition to (neuro)protection, MSCs' secretome may also support neural regeneration (Teixeira et al. 2013; Pinho et al. 2020; Cantinieaux et al. 2013; Martins et al. 2017; Cizkova et al. 2018; Park et al. 2010; Tsai et al. 2018; Haider et al. 2015; Guo et al. 2016). In SCI, the lesion causes the loss of neurons and glial cells, leading to high-level inflammation, demyelination, and pain (Teixeira et al. 2013; Pinho et al. 2020). MSC-derived secretome has already shown a neuroprotective role against apoptosis-activated macrophages

(pro-inflammatory M1 microglia toward inflammation-resolving M2 cells) and pro-angiogenic activity in vitro (Cantinieaux et al. 2013). Additionally, it has also been demonstrated that MSCs' secretome impacts axonal function, promoting their outgrowth through the action of trophic factors such as BDNF, HGF, and VEGF (Martins et al. 2017; Cizkova et al. 2018) and leading to the decrease of oxygen-glucose deprivation-induced cell damage (Park et al. 2010) and intensification of neuronal connections (Tsai et al. 2018). Cantinieaux's group reported that treatment with BM-MSCs led to an evident locomotor recovery in an experimental animal model. These results were supported by the pro-angiogenic tissue-protective effects of a cytokine cocktail (VEGF-A, VEGF-C, osteopontin, EG-VEGF/PK1, TAL1A, MMP-13, and FGF-BP, among others) present in the secretome (Cantinieaux et al. 2013). Corroborating with these results, studies have shown that injecting secretome into lesioned animals promotes neuroprotection, attenuation of cavity formation, and preservation of the spinal tracts. These observations correlated well with the recruitment of CD68+ cells with a concomitant reduction in oxidative stress and creation of an anti-inflammatory environment at either the lesion or parenchyma site (Haider et al. 2015; Guo et al. 2016). More recently, different groups have observed an overall motor recovery in SCI rats after treatment together with an increase in tissue sparing and axon density at the lesion site, which were remarkably correlated with the presence of GAP-43-positive axons, upregulation of Olig-2 and HSP70 protein levels, activation of autophagy, and lower levels of inflammatory cytokines (IL-2, IL-6, and TNF-α), respectively (Cizkova et al. 2018; Tsai et al. 2018).

The mere administration of the vesicular fraction, including MSC-derived exosomes, contribute to the direct neuroprotective/neuroregenerative effects in experimental SCI models. Different research groups have reported that treatment with exosomal fraction in vivo successfully modulated the microenvironment of spinal cord lesions by delivering anti-inflammatory and pro-angiogenic factors (Lankford et al. 2018; Liu et al. 2019). It has been evidenced that MSC-derived exosomes suppressed inflammation via the direct generation of immunosuppressive M2 macrophages (Lankford et al. 2018) and decreased TNF-α, Interleukin (IL)-1α, and IL-1β, inhibited nuclear translocation of NF-κB, and increased IL-10 secretion, thereby leading to neurotoxic A1 astrocyte suppression (Liu et al. 2019; Huang et al. 2017). Also, the attenuation of neuronal cell apoptosis was observed due to abrogation of pro-apoptotic proteins (Bcl-2-associated X protein, Bax, activated caspase-3, and caspase-9) and an upregulation in the levels of anti-apoptotic ones (i.e., Bcl2; B-cell lymphoma 2), which correlated well with axonal regeneration, glial scar suppression, mitigation of the lesion size, and a better functional behavioral recovery (Huang et al. 2017; Lankford et al. 2018; Liu et al. 2019). In a similar approach, Dong et al. and Zhou et al. attributed the modulatory effects to the miRNA content of exosomes claiming that both MSC-derived EVs miR-21-5p and miR-133b are able to activate distinct signaling mechanisms, thus influencing positively the treatment of SCI (Zhou et al. 2019; Li et al. 2018). Curiously, in a comparative study of secretome versus vesicles, Lu et al. have reported that the administration of BMSC-derived EVs could sustain the integrity of the blood-spinal cord barrier (BSCB) by increasing the total number of pericytes in the barrier

throughout the suppression of their migration (via the downregulation of NF-κB p65 signaling) (Lu et al. 2019).

Similarly, the effects of treatment with MSC-derived secretome have also been observed in ischemic stroke (IS) conditions. IS is caused by a reduction in blood supply that leads to a multicell signaling phenomenon englobing the death of endothelial and neuronal cells, inflammation (with prominence for microglia activation), and white matter pathophysiology (Pinho et al. 2020; Vizoso et al. 2017; Xing et al. 2012). When applied in IS models, Huang et al. reported the secretome competence to block astrocytic cell death, raising their metabolism through the action of IGF-1 and BDNF (Huang et al. 2015). Moreover, they correlated these outcomes to the inhibition of p38 MAPK and JNK signaling pathways (Huang et al. 2015). Similar to other pathologies addressed already, the therapeutic potential of MSC-derived exosomes has been shown in the context of IS as well. Besides functional recovery, exosome treatment post-injury provided an appropriate external milieu for successful brain remodeling since it enhanced axonal density and synaptophysin-positive staining, thereby providing long-term neuroprotection due to higher angioneurogenesis levels and immunosuppression (Xin et al. 2013; Lee et al. 2016a; Doeppner et al. 2015). In line with this, Xi and colleagues have shown that upon MSC-derived exosome treatment, the presence of neuroblasts and endothelial cells in ischemic regions was significantly increased, demonstrating MSC-derived exosomes as key players contributing toward neuronal differentiation (Xin et al. 2013). Additionally, a novel role of EVs has been reported in the myelination processes in IS. The authors have reported that after IS, EVs could restore white matter integrity by interfering in the axonal sprouting and growth, oligodendrocyte formation, tract connectivity, and remyelination (Otero-Ortega et al. 2017).

Neurodegenerative Disorders

Multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are the most common and well-known brain neurodegenerative pathologies with a higher incidence in the world's population (Baez-Jurado et al. 2019; De Pedro-Cuesta et al. 2015; Cannon and Greenamyre 2011). Although different pharmacological treatments and surgical procedures improve the quality of life for these patients, long-term clinical recovery is not achieved. Hence, the progressive degeneration continues, leading to the consequent loss of neurons (Baez-Jurado et al. 2019). In search of alternative treatments for these brain disorders, stem cell-free strategy using stem cells' secretome is also being considered (Drago et al. 2013). Various studies (both in vitro and in vivo) have described MSC-derived secretome as a stimulator of neurotrophic factor release (i.e., BDNF, a factor commonly affected in the majority of neurodegenerative disorders) and neuronal survival pathways (Vizoso et al. 2017) that allow the prevention of cell death (Drago et al. 2013; Fu et al. 2006) and promote neuronal cell survival, differentiation, and proliferation of pre-existing cells (Mita et al. 2015; Kim et al. 2015;

Moraes et al. 2012; Teixeira et al. 2015; Teixeira et al. 2017). The authors have also observed induction of endogenous neurogenesis and synaptic activity in the main affected areas (e.g., the hippocampus in AD or the substantia nigra in PD) (Kim et al. 2015; Teixeira et al. 2017; Cova et al. 2010; Wang et al. 2010). Secretome treatment also reduced the accumulation of protein aggregates such as alpha-synuclein in PD (Oh et al. 2017), misfolded tau protein (AT tau) and β-amyloid plaques in AD (Zilka et al. 2011; Kim et al. 2012), and mutant Huntingtin (mHtt) in HD (Lee et al. 2016b), which per se are focal hallmarks of these diseases. Lastly, cell-free therapy partially rescues the representative phenotype of the disability (Baez-Jurado et al. 2019; Pinho et al. 2020; Drago et al. 2013; Wang et al. 2010; Reza-Zaldivar et al. 2018; Sadan et al. 2009; Teixeira et al. 2020; Kim et al. 2014; Mendes-Pinheiro et al. 2020).

MSCs' secretome can incur behavioral improvements predominantly at the level of the motor and cognitive functions (Mita et al. 2015; Kim et al. 2014, 2015; Teixeira et al. 2015, 2017; Zilka et al. 2011; Vilaça-Faria et al. 2019; Mendes-Pinheiro et al. 2019). At organelle and molecular levels, there is significant modulation of oxidative stress, mitochondrial dysfunction, apoptosis, inflammation, and the activation of ubiquitin-proteasome system (Mita et al. 2015; Kim et al. 2014, 2015; Lee et al. 2016b; Reza-Zaldivar et al. 2018; Jarmalavičiūtė et al. 2015). Of particular interest were the results reported by Mendes-Pinheiro et al. using an experimental PD rat model. The authors reported that the administration of MSC-derived secretome led to a more efficient response when compared to the MSCs' transplantation in terms of preserving the dopaminergic system (Mendes-Pinheiro et al. 2018). In another exciting study, Katsuda et al. explored the use of MSC-derived exosomes in the context of AD. The authors have observed that after MSC-derived exosome delivery to an experimental animal AD model, there was a remarkable decrease in the levels of brain-soluble Aβ plates, a fact that was attributed to the presence of neprilysin in exosomes (Katsuda et al. 2013). Concerning HD, intrastriatally transplanted bone marrow-derived MSCs integrated well in the host brain. They showed trophic effects through an increase in laminin, von Willebrand factor (VWF), stromal cell-derived factor-1 (SDF-1) α, and the SDF-1 receptor CXCR4, which in turn enhanced angiogenesis in the damaged striatum (Lin et al. 2011). Finally, in experimentally induced demyelinating models such as MS, secretome application was found to elicit an effect in the oligodendrogenic niche of the subventricular zone that led to oligodendrogenesis and functional remyelination (Cruz-Martinez et al. 2016).

Clinical Approaches Based on Mesenchymal Stem Cell Secretome

As earlier discussed, pre-clinical experimental studies using MSC-derived secretome have given encouraging data, which has led to its applications in clinical settings. Nevertheless, the number of clinical trials around this topic is still scarce (Fig. 3). According to the US National Institutes of Health official database, only 45 secretome-based clinical trials have been reported as of February 21, 2021.

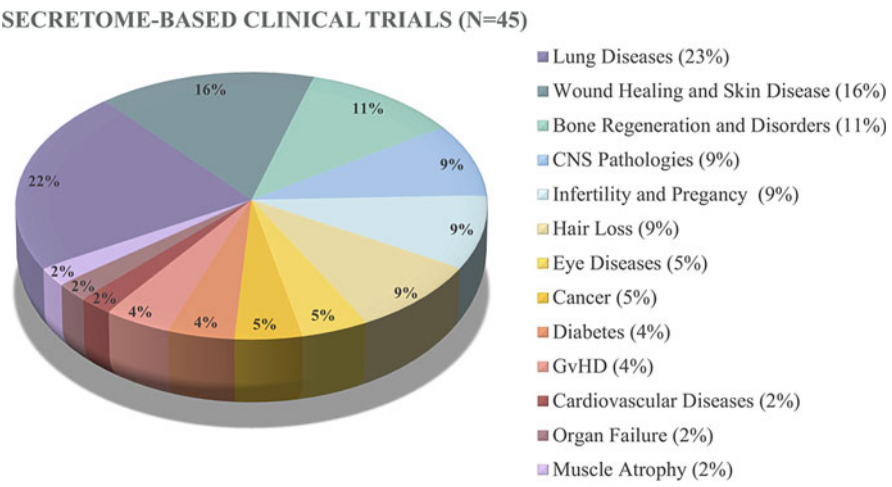

Fig. 3 Representation of the percentages of MSCs' secretome-based clinical trials. Data acquired from www.clinicaltrial.gov

Most of these trials were primarily aimed to assess the biomedical potential of either secretome or their derivatives (e.g., exosomes), addressing their safety, feasibility, and efficiency in improving the clinical outcomes of the participating patients. Secretome translation from the bench to the bedside has been mainly covering the treatment of conditions like graft-versus-host disease (GvHD), hair loss, COVID-19 pneumonia, diabetes, cancer, infertility, as well as lung, cardiovascular, bone and cartilage, skin, neurological, and autoimmune diseases (Fig. 3) (NCT04213248, NCT02192736) (Zhou et al. 2013a; Fukuoka and Suga 2015; Shin et al. 2015; Katagiri et al. 2016; Kordelas et al. 2014; Dahbour et al. 2017).

Notably, the employment of secretome in COVID-19-associated pneumonia therapy has boomed in the preceding year, with seven clinical trials reported on http://www.clinicaltrials.gov (last access: 21/02/21). Herein, the conjoint idea relies on investigating the effects of intravenous injection/inhalation of secretome components (especially exosomes) for the treatment of patients with moderate/severe SARS-CoV-2 infection (NCT04753476; NCT04276987; NCT04602442; NCT04491240; NCT04398303; NCT04747574) and also to explore their tolerance in healthy volunteers (NCT04313647) (Abraham and Krasnodembskaya 2020; Bari et al. 2020).

The administration of secretome in the form of conditioned medium in clinical context has been illustrated in patients suffering from severe alveolar bone atrophy, alopecia (or hair loss), MS, and skin injury. In the completed clinical studies, the use of ADSC-derived secretome in alopecia and female pattern hair loss (FPHL) could restore hair density, revealing itself as a novel therapy for hair regeneration (Fukuoka and Suga 2015; Shin et al. 2015). In the case of alveolar bone regeneration, upon MSC-derived secretome administration, patients needing bone augmentation revealed mineralization, early bone formation, and reduced inflammatory signs.

In this first-in-human clinical study, researchers demonstrated the safety of secretome as a potential modulator of osteogenic regenerative medicine (Katagiri et al. 2016). Similarly, in an open-label prospective phase I/II clinical study, MSC-derived secretome was used for the first time to treat MS patients. The authors reported an improvement in all the measuring tests and observed a correlation between the decrease of the lesion and increased IL-6, IL-8, and VEGF contents in the secretome and concluded that the intervention was clinically safe and feasible (Dahbour et al. 2017).

Currently, the focus has shifted more to the clinical applications of MSC-derived exosomes. Several ongoing clinical trials have already reported the benefits of exosome-based therapy (http://www.clinicaltrials.gov). For instance, a preliminary study showed that the application of a specific regimen of MSCs' derivatives in a GvHD patient (with skin and intestinal tract complications) yielded a significant and sustainable improvement of symptoms by reducing the release of inflammatory cytokines and attenuating the ongoing inflammation in the gut and skin (Kordelas et al. 2014). Additionally, they noticed that the treatment was well-tolerated and remained stable for 5 months, denoting a long-lasting therapeutic influence of the exosomes (Kordelas et al. 2014). Furthermore, based on the available initial reports, it is predictable that further trials would be initiated soon. In light of the already completed human clinical studies exploiting secretome's activity, it seems that they have established some degree of safety and viability. In fact, no data has been presented relevant to any adverse effects, demonstrating that the delivery (local or systemic) of MSCs secretome as whole and/or MSCs-derived exosomes in patients with distinct illnesses might be a secure and viable therapeutic approach in the future.

The Benefits and Barriers of MSCs' Secretome

In this chapter, we have shown that secretome-derived products are appropriate to significantly improve numerous pathophysiological mechanisms in experimental animal models of diseases as well as clinical trials approved by national agencies. Exploiting MSC-derived secretome and its insoluble derivatives (i.e., vesicles, exosomes) in experimental and clinical settings offers several benefits to the domain of stem cell-based approaches and regenerative medicine. Firstly, when using secretome, it is possible to solve some concerns related to the transplantation of living and proliferative cell populations, such as overcoming the low cell survival that follows the procedure, reducing the large number of cells needed, providing less immunogenicity and tumorigenicity, and decreasing the possibilities to alter its phenotypic and therapeutic potential as observed with MSCs' expansion in vitro (Vizoso et al. 2017; Teixeira and Salgado 2020). Moreover, MSC-derived secretome also possesses advantages in terms of manufacturing, storage, management, and product shelf-life (Teixeira et al. 2016). Secondly, time and cost of expansion/maintenance of cultured stem cells in dynamic culture conditions (e.g., bioreactors) can be greatly reduced. Thirdly, due to the nonliving profile of the secretome,

this can be easily stored without the use of potential toxic cryoprotectant agents, packaged, and safely transported without losing its effectiveness, which represents a critical statement for the economic viability. Fourthly, the evaluation of secretome safety, dosage, and potency may be much simpler and analogous to the regular pharmaceutical compounds than the cells; and finally, being deemed as a ready-to-go product implies that it is immediately available for treatment (Baez-Jurado et al. 2019; Vizoso et al. 2017; Teixeira and Salgado 2020; D'angelo et al. 2020; Praveen Kumar et al. 2019).

Nevertheless, although promising, the translation of MSC-derived secretome to clinical practice remains a challenge. Unfortunately, defining the complete biochemical composition of the secretome is difficult as it is altered by many factors and involves multiple mechanisms, which are responsible for its beneficial properties (Pinho et al. 2020; Vizoso et al. 2017; Teixeira and Salgado 2020; D'angelo et al. 2020; Mendes-Pinheiro et al. 2020). Actually, a relevant issue that remains elusive is the identification and characterization of the secretome-derived extracellular vesicles and their contents (Abraham and Krasnodembskaya 2020; Drago et al. 2013; Basu and Ludlow 2016). Moreover, when using secretome, it is often hard to measure the activity and half-life of its components, as well as to access their pharmacokinetics, bio-distribution, tissue transport, and protein stability (Pinho et al. 2020; Teixeira and Salgado 2020).

Improvements Needed for Secretome Application as a Cell Transplantation-Free Tool

As soon as we understand and interpret the diverging data of using multiple conditions and platforms, we will be of guarantee a homogenous, scalable, and applicable secretome product (Teixeira and Salgado 2020). Undeniably, to attain a true impact, concomitant progress in multiple fields has to be achieved (Makridakis et al. 2013). Therefore, as a way to understand and boost secretome applications, a complete characterization of MSC-derived secretome becomes vital not only to identify the full scope of biochemical factors (with a special focus on active molecules that may be oncogenic) but also to clarify if the molecules released can target the cell and tissue dynamics. Likewise, it is also essential to elucidate the molecular mechanisms and pathways underlying the secretome-mediated effects (Marques et al. 2018; Mendes-Pinheiro et al. 2020). For instance, thorough proteomic analysis, next-generation metabolomics-driven approaches, and system biology techniques (Drago et al. 2013) may contribute immensely to understanding the secretome profile of cells cultured under diverse conditions. At the same time, regulatory requirements for manufacturing and quality control must still be implemented (Pinho et al. 2020; Vizoso et al. 2017). Standardized protocols for secretome production, storage, and transport should be employed to limit heterogeneity and enrich the predictability of secretome-derived products. Linked to this, it will also be crucial to define an optimal timing for secretome collection (Teixeira and Salgado 2020), as well as a bio-manufacturing protocol for collection (Abraham and

Krasnodembskaya 2020; Vizoso et al. 2017). Another important step forward should be the modulation of secretome and/or EV composition to create an optimal biocomponent and to personalize it according to each patient's condition. Finally, it is essential to highlight that before its application in clinical practice, additional studies should be performed paying attention to the treatment regimen, including the route and frequency of administration, the volume of injection, and dose application timeline (Praveen Kumar et al. 2019; Mendes-Pinheiro et al. 2020). Additionally, group-to-group data sharing and comparisons must be performed as a way to bring the field closer to clinical applications and, at the same time, help to understand secretome interactions with diverse pathologies in a global perspective (Abraham and Krasnodembskaya 2020) (Makridakis et al. 2013; Pinho et al. 2020; Teixeira and Salgado 2020; Mendes-Pinheiro et al. 2020). Doing so, this can lead to the rational design of multiple secretome strategies to tackle dissimilar pathophysiological deficits in a multifaceted way.

Conclusion

Recently, MSCs' secretome has emerged as a hopeful therapeutic tool to the regenerative medicine field. Apart from differences in their sources and composition, the secretome has been demonstrating therapeutic advantages compared to the classical cell-based approaches. Nevertheless, standardized parameters in the secretome production and its complete characterization are still missing, which somehow has delayed its consideration by the regulatory agencies and, consequently, its translation to the clinics.

Nevertheless, the secretome is already implied in clinical trials, demonstrating promising effects on various tissues and a plethora of targeted diseases. Therefore, the challenge for the years to come will be to understand the precise impact of the secretome in a targeted cell/area and make it the most suitable therapy for an issue by modulating it according to the biological/therapeutic necessity.

Cross-References

- ▶ Adipose Tissue-Derived Regenerative Cell-Based Therapies: Current Optimization Strategies for Effective Treatment in Aesthetic Surgery
- ▶ Advances, Opportunities, and Challenges in Stem Cell-Based Therapy
- ▶ Current State of Stem Cell Therapy for Heart Diseases
- ▶ Direct Reprogramming Strategies for the Treatment of Nervous System Injuries and Neurodegenerative Disorders
- ▶ Extracellular Vesicles Derived from Mesenchymal Stem Cells
- ▶ Mesenchymal Stem Cell-Extracellular Vesicle Therapy in Patients with Stroke
- ▶ Mesenchymal Stem Cells

► Sources and Therapeutic Strategies of Mesenchymal Stem Cells in Regenerative Medicine
► Stem Cell Applications in Cardiac Tissue Regeneration
► Therapeutic Uses of Stem Cells for Heart Failure: Hype or Hope

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Exosome-Based Cell-Free Therapy in Regenerative Medicine for Myocardial Repair

34

Khawaja Husnain Haider and Mustapha Najimi

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Abstract

Exosomes in cardiovascular theranostics are gaining popularity due to their immense diagnostic and therapeutic potential generally ascribed to their specific payload and temporospatial release under a specific set of conditions. A recently published bibliometric research analyzing 1039 studies has shown a flourishing trend in cardiovascular medicine. Exosomes constitute an integral part of the paracrine secretions of stem/progenitor cells, which significantly contribute to intercellular communication by transferring signaling molecules between communicating cells. Interestingly, their payload can be modified by the physical,

K. H. Haider (✉)
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

M. Najimi
Laboratory of Pediatric Hepatology and Cell Therapy, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium
e-mail: mustapha.najimi@uclouvain.be

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pharmacological, or genetic manipulation of the parent cells from which they have been derived. Alternatively, more recent studies have developed protocols to load them with the payload of interest for specific therapeutic applications. Similarly, they are also released from various myocardial cells, including cardiomyocytes, in response to injuries with diverging payload profiles, which can be used for diagnostic purposes. This chapter discusses in-depth mesenchymal stem cell (MSC)-derived exosomes for their theranostic application in cardiovascular pathologies encompassing in vitro, small experimental animal models as well as translational and clinical studies.

Keywords

Cell-free therapy · Exosomes · Heart · Infarction · Paracrine · Stem cells

Abbreviations

AMI	Acute myocardial infarction
BM	Bone marrow
CABG	Coronary artery bypass grafting
CSCs	Cardiac stem cells
CXCR4	Chemokine receptor-4
DOXO	Doxorubicin
ECs	Endothelial cells
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
GLUT	Glucose transporter
GMP	Good manufacturing practice
GP	Glycogen phosphorylase
Hsp	Heat shock protein
HUVECs	Human umbilical vein endothelial cells
IL	Interleukin
IONP-MSCs	Iron oxide nanoparticle loaded MSCs
iPSCs	Induced pluripotent stem cells
LV	Left ventricle
miR	MicroRNA
MSCs	Mesenchymal stem cells
PCI	Percutaneous coronary intervention
PEP	Purified exosome preparation
SDF-1α	Stromal-cell-derived factor-1α
Sfrp-2	Secreted frizzled protein-2
STG	Shear thinning hydrogel
TIMP-2	Tissue matrix metalloproteinase inhibitor-2
TNF-1α	Tumor necrosis factor-1α

Introduction

Cell-Based Therapy for the Heart

Based on encouraging data from small experimental animal models and translational studies, the use of stem-/progenitor-cell-based therapy has shown safety and feasibility in repopulating an infarcted myocardium with functionally competent neomyocytes and in reversing myocardial injury to preserve global contractile heart function. Notable and most extensively studied cell types used for cell-based therapy include bone marrow (BM)-derived mesenchymal stem cells (MSCs), BM-derived mononuclear cells, endothelial progenitor cells (EPCs), skeletal myoblasts, cardiac stem/progenitor cells (CSCs/CPCs), adipose tissue-derived MSCs, embryonic stem cells (ESCs) and their derivative cardiomyocytes, and, more recently, induced pluripotent stem cells (iPSCs) and their derivative cardiomyocytes and EPCs. Each one of the cell types has specific advantages and limitations and is still being scrutinized to find an ideal cell type, which could offer the best compromise between limitations and advantages (Young and Schäfer 2015). Among all these cell types, MSCs have already progressed to the advanced phases of clinical assessment in diverse patient populations. According to a recently published systematic review, there are 1138 clinical trials have been registered on ClinicalTrials.gov, of which 60% are phase II while 30% are phase I trials with BM as the primary source of MSCs in most of the clinical trials and all of the trials reporting the safety of the procedure (Rodríguez-Fuentes et al. 2020).

Irrespective of the cell type used, the stem-cell therapy approach is undoubtedly superior to contemporary treatment approaches in addressing the repair of the injured parenchyma of a cardiac architecture to limit the vicious progression of the disease process. It compensates for the loss of functioning cardiomyocytes, something not offered by any other treatment modalities. However, the further progression of the cell-based therapy approach is happening at a slower pace due to many factors encompassing the cell quality to meet the clinical-grade good manufacturing practice (GMP)-certified cell preparations with reproducible composition, to their biological characteristics, and uniform functionality, all of which remain problematic and time-intensive proposition (Bettonville et al. 2021). For further reading on the topic, please refer to ▶ Chap. 10, "Mesenchymal Stem Cells for Cardiac Repair," contributed by AlKhani et al. in this volume, which provides a detailed account of the subject relevant to the experience of working with MSCs from small animal experimental models to large experimental animal models and in the randomized clinical trials in heart failure patients. Similarly, for the quality aspects of cell preparation, please refer to a recently published literature review, which discusses in depth the issue of the quality of cell preparation and the possible areas of improvement therein (Haider 2018).

Cell-Free Therapy Approach for the Heart

As discussed earlier, cell-based therapy has shown promise both in experimental animal models and clinical settings. Despite all the advantages, cell-based therapy has some limitations, which have significantly slowed its progress to the clinics. For example, the extensive death of donor cells, especially during the acute phase of engraftment; limited differentiation; the integration of the capacity of the transplanted cell; etc. severely hamper the desired outcome. Although various protocols have been tried and developed, we are still lacking in expertise to eliminate these issues in toto. For example, to overcome the issue of extensive donor cell death, protocols ranging from transient immunosuppression to the preconditioning of donor cells have been developed, tried, and reported, but the problem continues to persist (Haider et al. 2004; Xiao et al. 2004; Haider and Ashraf 2012). On the same note, the priming of cells through genetic modifications, cytokine and growth factor treatment, pharmacological and small molecule treatment, etc. has been investigated to enhance the cardiogenic differentiation of stem/progenitor cells (Noronha et al. 2019; Ullah et al. 2021); however, there are still many limitations on a clinical perspective.

The paracrine activity of stem cells, which involves the release of both soluble and insoluble factors, constitutes an integral part of their therapeutic benefits post engraftment in the injured heart (Haider and Aziz 2017; Lei and Haider 2017). Based on the paracrine activity of stem cells, researchers have, more recently, started to focus on the cell-free therapy approach to avoid some of the potential limitations of cell-based therapy, such as difficulties in the clinical-grade manufacturing of cells, prevention of a likely tumorigenic potential, transmission of infection from the cell donor, immune rejection of the cells and cell-derived tissue graft, possible undesired lineage differentiation or no differentiation of the donor cells, etc. (Haider and Aslam 2018). Furthermore, the cell-free therapy approach also alleviates the need for in vitro cell expansion to achieve millions of cells needed for cell-based therapy. A modest outcome from the completed and ongoing clinical trials necessitates alternative approaches for a better therapeutic outcome (Haider 2018).

As discussed earlier, the soluble and insoluble components of the cell secretome are rich in bioactive molecules (Rahimi et al. 2021). Although both secretome components have given encouraging results in the experimental animal models, the current focus is on extracellular vesicles (EVs) in general and exosomes in particular. The interest of researchers is directed toward exosomes in regenerative medicine, as may be judged by the fact that the keyword search on Pubmed yields more than 15,000 publications, during the last 5 years, primarily focused on their theranostic applications.

Theranostic Role of Exosomes in the Heart

Although exosomes of plant and human tissue origin have progressed for clinical assessment, the use of human-tissue-derived exosomes is much more established in terms of their biological characterization, payload analysis, and GMP compliance.

For their physical and biological characteristics and cell-specific payload, they are emerging as promising tools for cardiovascular theranostic applications (Wumei et al. 2021; Jayaraman et al. 2021). However, for clinical applications, exosomal preparations should conform to the minimum standards of GMP. Various research groups and drug manufacturing companies are coming up with their optimized protocols to achieve GMP in the manufacturing of exosomes to ensure high exosome yield with minimum levels of extraneous materials, i.e., xenogenic proteins, serum-derived exosomes, etc. (Pachler et al. 2017; Andriolo et al. 2018). However, the Food and Drug Administration (FDA) has yet to approve any of these exosome preparations for routine clinical use. Many details about the characteristics, properties, and cargo of exosomes are also available through online sources and databases, like ExoCarta (http://www.exocarta.org), ExoBCD (https://exobcd.liumwei.org), Vesiclepedia (http://microvesicles.org), EVpedia (http://evpedia.info), exoRBase (http://www.ExoRBase.org), and exRNA Atlas (https://exrna-atlas.org), which provide bioinformatics-based information in a comprehensive and detailed manner (Sahoo et al. 2021). These online resources provide information seekers and researchers with an update on exosome and EV biology and an analysis of their payload.

Diagnostic Applications of Exosomes

Like any other cell, cardiac cells, i.e., cardiomyocytes, telocytes, CPCs, etc., also release exosomes with specific payloads and functions (Vrijsen et al. 2016; Liao et al. 2021). For example, the profiling of cardiomyocyte-derived exosomes has revealed the presence of miR-208a, which gets transferred to the cardiac fibroblasts as a mechanism for cross talk between the two cell types to initiate myocardial fibrosis in the event of myocardial injury (Yang et al. 2016). Similarly, uptake of the injured cardiomyocyte-released exosomes by the transplanted bone marrow MSCs in the infarcted mouse heart has been shown to initiate cell-to-cell communication resulting in loss of the transplanted cell. This problem has significantly hampered the progress of cell-based therapy (Hu et al. 2018). These in vivo data were supported by MSCs cultured in vitro in the conditioned medium and exosomes derived from cardiomyocytes cultured under oxidative stress. Molecular studies revealed significantly high caspase-3 activation and decreased Bcl2/Bax in MSCs, which showed more elevated apoptosis under oxidative stress.

On the other hand, Ribeiro-Rodrigues and team have demonstrated that cardiomyocytes cultured under ischemic conditions released exosomes, which had angiogenic potential when added to cultured endothelial cells (ECs) (Ribeiro-Rodrigues et al. 2017). ECs cultured in the presence of H9C2 and primary cardiomyocyte-derived exosomes promoted EC proliferation, protection against oxidative stress, and enhanced tubulogenesis as compared to normal cardiomyocyte-derived exosomes. The profiling of exosomes from cardiomyocytes cultured under ischemic conditions showed that they were rich in metalloproteases and miR-143 and miR-222, which might be attributed to their enhanced angiogenic potential.

In summary, the cardiomyocyte-released exosomes are integral mediators of their communication with other cells through the inclusion of specific payloads, such as heat shock proteins (Hsp)-20, 60, and 70, which regulate their apoptosis and survival; inflammatory factors IL-6 and TNF-a, which are responsible for cardiac remodeling; GLUT1, GLUT4, and glycolytic enzymes, which are involved in glucose transport and metabolism; and a long list of miRs, which regulate various cellular functions (Yu and Wang 2019).

Given the significant role of EVs as mediators of intercellular communication, various research groups have focused their research on defining a role for cardiomyocyte-derived exosomes released as biomarkers of myocardial injury (Doran and Voora 2016). They have predominantly focused on the analysis of differential exosomal payload changes in response to myocardial injury. Recent advances are directed toward using a liquid biopsy approach, which involves sampling and the analysis of biofluids to detect the presence of exosomes with a specific payload for diagnostic applications (Valencia and Montuenga 2021).

In the cells of the cardiovascular system, such as cardiomyocytes, ECs, smooth muscle cells, and other stem/progenitor cells in the heart, exosomes have a significant role in development, injury, and disease progression (De Freitas et al. 2021). A recently published systematic review has published the commonalities and differences of exosomes and their payloads in the six different types of cardiac-muscle-derived cells (Xu et al. 2019). In this regard, exosomal miR payload may have remarkable diagnostic and prognostic applications and is being exploited as a biomarker (Zamania et al. 2019). Moreover, the number of exosomes and the cell type of their origin significantly contribute to their diagnostic and prognostic value (Zarà et al. 2020; Tian et al. 2021). An example is the quantification of released EVs in response to doxorubicin (DOXO)-induced cardiac injury in an experimental mouse model (Yarana et al. 2018). The authors of the study observed a significant increase in the number of circulating EVs released in response to DOXO-induced injury.

Moreover, the released EVs were rich in liver, heart, and muscle-related isoforms of glycogen phosphorylase (GP), while treatment with cardioprotective agents significantly reduced the EV payload of GPs. Beaumir et al. studied altered EV-miR payload as a potential biomarker of cardiotoxicity in nine client-owned dogs, which received five doses of DOXO therapy for sarcoma (Beaumier et al. 2020). The authors observed significant changes in at least ten EV-derived miRs, among which miR-502 showed a substantial increase while miR-107 and miR-146a recorded a considerable reduction in expression levels.

Deddens et al. have reported spatiotemporal release characteristics of EVs in response to myocardial ischemia (MI)/reperfusion injury and profiled their miR payload in experimental murine and porcine models (Deddens et al. 2016). They observed the release of muscle-specific miRs (myomiRs), i.e., miR-1, miR-133b, miR-208b, and miR-499, in plasma after ischemia-reperfusion injury (miR-499 showed the highest levels in the plasma after MI injury). Their plasma levels were well correlated with cardiac Tn-I release, a well-established biomarker of myocardial injury (Haider and Stimson 1993). Interestingly, both miRs and exosomes appeared

simultaneously in the plasma samples upon cardiac injury, and exosomes were found rich in miR-133b, miR-208b, and miR-499 but not miR-1. A position paper by the working group on the cellular biology of the heart provides an excellent review of literature on the topic for further reading (Sluijter et al. 2018).

Exosomes as a Treatment Option for the Injured Heart

Exosomes derived from various stem/progenitor cells, i.e., ESC-derived cardiovascular progenitors, EPCs, iPSCs, adipose-tissue-derived MSCs, etc., have been extensively investigated for their therapeutic potential for myocardial repair (Barile et al. 2014; Cui et al. 2017; Chen et al. 2018; Kurtzwald-Josefson et al. 2020; Wu et al. 2020; Chung et al. 2020). A recently published systematic review based on the analysis of 24 experimental animal studies revealed that MSC-derived exosome treatment significantly improved cardiac function due to enhanced angiogenic response in the infarcted myocardium, besides reduced cell death (Meng et al. 2021).

Exosome in Experimental Animal Heart Models

The cytoprotective effects of MSC-derived exosomes have been investigated in many recently published experimental animal studies on myocardial damage (Table 1). Liu et al. used exosomes derived from naïve MSCs to rescue ischemia-reperfusion injury to neonatal cardiomyocytes in vitro and post intramyocardial injection in an experimental rat heart model of ischemia-reperfusion injury (Liu et al. 2017). The authors observed significantly attenuated myocardial injury. Molecular mechanistic studies in vitro showed internalization of exosomes by the cardiomyocytes and rescued them from H_2O_2 oxidative stress by inducing their autophagy via activation of AMPK/mTOR and Akt/mTOR signaling pathway (Liu et al. 2017). In a similar study, treatment with bone marrow MSC-derived exosomes revealed the repression of PTEN in H2C9 cells via miR-486-5p and the activation of PI3K/Akt signaling, which rescued the cells from hypoxia/reoxygenation injury (Sun et al. 2019). Zhao et al. have reported that MSC-derived exosomes via miR-182 mediated the polarization of M1 macrophages to M2 macrophages both in vitro and in vivo, with toll-like receptor 4 as its downstream target (Zhao et al. 2019).

Besides other biomolecules, MSC-derived exosomes show a differential expression of both the negative, i.e., miR-130, miR-378, and miR-34, and positive, i.e., miR-29 and miR-24, regulators of cardiac function as their miR payload can be altered according to the intended outcome (Shao et al. 2017; Bellayr et al. 2017). Hence, MSCs have been manipulated in vitro to alter their miR payload for mircrining the injured heart using exosomes as carriers of the desired miRs (Haider and Aramini 2020). For example, MSCs have been genetically modified for myomiRs (myocardium-related miRs), i.e., miR-1, miR-133, miR-208, and miR-499, to enhance their cardiac differentiation (Huang et al. 2013; Lee et al. 2013; Neshati et al. 2018). Various research groups have assessed the effect of genetically modified MSC-derived exosomes for miR-125b expression on

Table 1 A summary of preclinical trials utilizing bone-marrow-derived MSCs in the small animal models of heart diseases

Author/year	Source of exosomes	Delivery mode	Model type	Animal	Main findings
Sun et al. (2018)	BM MSC-derived exosomes	I/V injection	Doxorubicin-induced cardiomyopathy	Mice	Treatment with MSCs reduced cardiomyocyte apoptosis, reduced inflammatory cell infiltration as compared to the PBS-treated control group, and significantly reduced macrophage infiltration in the infarcted myocardium, JAK/STAT pathway activation. A significantly reduced infarct size and improved cardiac function were observed
He et al. (2018)	Exosomes derived for mouse BM-derived MSCs overexpressing GATA4	Intramyocardial injection	MI	Mice	BM-derived MSCs were transduced with a lentiviral vector encoding for GATA4 and cultured for exosome collection. Exosomes protected cardiomyocytes in vitro under hypoxic stress, while treating MSCs with exosomes increased cardiac marker expression and protected them against anoxic stress. It significantly increased LVEF and LVFS posttreatment in the infarcted rat heart, with increased blood vessel density, increased cardiac c-kit+ cell homing, and reduced cardiomyocyte apoptosis
Xiao et al. (2018)	Human BM-derived MSCs	I/M	MI	Mice	There was a significant reduction in autophagic flux and death in neonatal cardiomyocytes, under hypoxia, cultured with MSC-derived exosomes and p53-Bnip3 signaling. These changes were abolished by exosomal inhibitor GW4869 and the inhibition of miR-125. In vivo treatment in a mice model of MI significantly improved infarct size and cardiac function, which were abolished by exosomes derived from MSCs treated with miR-125
Ma et al. (2018)	Mice BM MSCs-derived exosomes loaded with miR-132	I/M injection	MI by LAD ligation	Mice	Internalization of miR-132-loaded exosomes by the incubated HUVECs with the reversal of its target gene RASA1 significantly increased tube formation. RASA1 was confirmed by luciferase assay as the downstream target of miR-132. Treatment of mice with infarcted hearts led to increased neovascularization in the peri-infarct area and preserved global cardiac function

Zhao et al. (2019)	Mouse BM-derived MSCs		I/R injury	Mice	MSC-derived exosomes were injected I/M immediately after I/R injury. It suppressed inflammatory response. There was a significant reduction in systemic macrophages. There was a significant modification of M1 macrophages to M2 macrophages in vivo and in vitro with the vital role of miR-182
Bian et al. (2014)	Hypoxia-treated human BM-derived MSCs	I/M	MI	Rat	The study used the therapeutic efficacy of exosomes from hypoxia-treated human BM-derived MSCs. In vitro studies showed rapid internalization of exosomes by cultured HUVECs, which showed increased migration, proliferation, and tubulogenesis. I/M injection of exosomes significantly reduced fibrosis and infarct size and increased regional blood flow recovery due to angiogenesis. Overall, there was preserved cardiac function
Yao et al. (2021)	MSC-derived exosomes	Spray formulation	AMI	Mice	Designing, fabrication, and testing of a spray of exosomes in biomaterial – the treatment significantly improved cardiac function, reduced cardiac fibrosis, and promoted endogenous angiomyogenesis in the postinjury heart
Shao et al. (2017)	Rat BM-derived MSCs	I/M	MI	Rat	The study compared the efficacy of MSCs and their derived exosomes for myocardial repair. Treatment with exosomes significantly reduced myocardial fibrosis and inflammation and recovered cardiac function. In vivo studies showed MSCs and their derived exosomes had similar miRNA profiles. Also, exosome treatment of H2C9 increased their proliferation, protecting them against oxidative stress, and inhibited TGF-b-induced transformation of fibroblasts to myofibroblasts
Ma et al. (2017)	Human umbilical cord-MSCs with or without expressing Akt-derived exosomes	I/V injection after coronary artery ligation	AMI	Rats	Endothelial cell function, i.e., proliferation, migration, and tubulogenesis, increased in vitro upon treatment with Akt-MSC-derived exosomes. Akt-exosomes were rich in PDGF-D. Treatment of MI with Akt-exosome significantly improved cardiac function mediated by myocardial angiogenesis

(continued)

Table 1 (continued)

Author/year	Source of exosomes	Delivery mode	Model type	Animal	Main findings
Huang et al. (2019)	Atorvastatin-treated rat BM-derived MSCs & MSC exosomes	I/V delivery of cells & I/M delivery of exosomes	Acute MI	Rat	I/M delivery of BM-derived MSC exosomes without or combined with I/V injection of atorvastatin-treated MSCs on day 1, 3, or 7. Echocardiography showed the most significant improvement in cardiac function, reduced infarct size in the combined treatment group receiving cells on day 3 after AMI. This was assigned to increased neovascularization, higher recruitment of MSCs, and reduced inflammatory response due to exosome treatment, which also reduced apoptosis of the recruited cells
Lv et al. (2019)	Three to five passages Rat BM-MSC-derived exosomes in alginate gel	I/M injection 30 min after ligation	MI by LAD ligation	Rat	I/M injection of MSC-derived exosomes in hydrogel enhanced their retention at the site of injection. There was a significant reduction of cardiomyocyte apoptosis and polarization of macrophages on day 3 after injection. At 4 weeks after delivery, scar thickness increased, heart function improved significantly in the hydrogel-exosome-treated group of animals
Cheng et al. (2020)	MSCs, MSC-exosomes, exosomes from MSCs cultured in GW4869, & MSCs with anti-miR-210	I/M	MI	Rat	The study determined the role of miR-210 in cardioprotection by exosomes derived from MSCs through a gain- or loss-of-function approach. MSC-derived exosomes significantly protected cardiomyocytes under hypoxia. Direct I/M injection of exosomes significantly reduced infarct size and area of fibrosis, with the significant role of miR-210 as a mediator of therapeutic outcome and AIFM3 as a downstream target

Abbreviations: *AMI* acute myocardial infarction, *BM* bone marrow, *miR* microRNA, *I/M* intramyocardial, *I/V* intravenous, *LAD* left anterior descending artery, *MI* myocardial infarction, *MSCs* mesenchymal stem cells, *p53-Bnip3* B-cell lymphoma 2-interacting protein 3, *PDGF-D* platelet-derived growth factor

ischemia/reperfusion injury in the heart (Chen et al. 2019, 2020; Qiao et al. 2019), which was previously shown to reduce cardiomyocyte apoptosis due to ischemia/reperfusion injury via tumor necrosis factor receptor-associated factor (TRAF)-mediated activation of NF-kb (Xiaohui et al. 2014). The authors observed that intervention with miR-125b carrying exosomes increased cell viability and reduced apoptotic ratio with the concomitant downregulation of Bax and caspase-3 and the upregulation of Bcl2. Although the miR-centric explanation of the beneficial effects has gained vast acceptance among researchers in the field, some researchers have challenged this due to the low copy number of miRs present in the exosomes and attributed the beneficial effects to a protein-centric explanation based on the presence of enzymes, growth factors, etc. (Eisenstein 2020).

Although the data from naïve MSC-derived exosomes have been encouraging, various strategies have been adopted to accentuate the therapeutic benefits of MSC-derived exosomes by manipulating their payload for delivery. This can be achieved by directly loading the payload of interest into the exosomes or alternatively by manipulating the cells from which the exosomes are to be derived. The latter strategy involves the physical, genetic, chemical, or pharmacological manipulation of the cells in culture to enhance the rate of exosome release as well as to modify their payload to achieve the desired benefits. For example, Bian et al. used hypoxia-treated MSC-derived vesicles of 100-nm size for the treatment of experimentally infarcted rat heart (Bian et al. 2014). The intramyocardial injection of vesicles significantly enhanced angiogenic response in the infarcted heart, which led to improved regional blood flow, reduced infarct size, and improved diastolic performance. The data were supported by in vitro studies during which vesicles were internalized by cultured human umbilical vein endothelial cells (HUVECs), which showed enhanced proliferation, migration, and tubulogenesis. The authors inferred that treatment with MSC-derived vesicles (a mix of exosomes and microvesicles) had cardioprotective effects through enhanced angiogenic response. To further dissect the mechanism, Wang et al. analyzed hypoxia-treated MSC-derived EVs for their payload and observed that the EVs were rich in miR-210 (Wang et al. 2017). The abrogation of miR-210 significantly abrogated EV angiogenic potential in vitro and in vivo in a murine heart model of acute myocardial infarction (AMI). It is pertinent to mention that hypoxamir-210, a member of the hypoxia-inducible factor-1a-dependent miR family (Haider et al. 2009), is overly expressed in hypoxia-preconditioned MSCs, the transplantation of which resulted in an angiomyogenic repair of the infarcted rodent heart post engraftment, besides cytoprotection (Kim et al. 2009, 2012a, b). A summary of the exosome-based treatment strategy for myocardial infarction is provided in a recently published review of literature by Wang et al. (2021).

Alternatively, the genetic manipulation of MSCs for the overexpression of various genes has been shown to significantly alter the payload of their derivative exosomes and hence their therapeutic benefits in treating infarcted myocardium. For example, Liu et al. genetically modified MSCs to overexpress macrophage migration inhibitory factor (MIF) and later cultured the cells for exosome collection (Liu et al. 2020). The treatment of neonatal cardiomyocytes with exosomes

significantly reduced their apoptosis as well as mitochondrial fragmentation under serum-/nutrient-deprived culture conditions. The intramyocardial injection of exosomes significantly restored myocardial function in an experimental rat heart model of myocardial infarction and caused significantly reduced cardiomyocyte apoptosis in the recipient rat hearts.

Ni et al. genetically modified MSCs with tissue matrix metalloproteinase inhibitor-2 (TIMP-2), a significant determinant of myocardial remodeling after MI (Ni et al. 2019). The genetically modified cells were cultured in vitro for exosome collection, which was then successfully used for treating experimentally infarcted rodent hearts. The intramyocardial injection of exosomes at multiple sites in and around the infarcted zone increased Akt expression, which was related to the high-level expression of secreted frizzled protein-2 (Sfrp-2) in the TIMP-2 overexpressing MSC-derived exosomes, resulting in reduced cardiomyocyte apoptosis, decreased oxidative stress, and remodeling with the involvement of Sfrp-2/Akt signaling. Some of the other transgenes used to modify MSCs to obtain exosomes include Akt, SIRT1, GATA4, CXCR4, SDF-1α, etc. (Ma et al. 2017; Kang et al. 2015; He et al. 2018; Gong et al. 2019; Huang et al. 2020b). Most of these studies have reported a single transgene overexpression strategy in the cells. It would be interesting to characterize the cells for the payload profiling of their derivative exosomes after multitransgene modification to observe how the cells adjust for multitransgene overexpression in terms of exosome payload composition (Konoplyannikov et al. 2013).

The strategy of pharmacologically manipulating MSCs to alter their derived exosomal contents has also been reported by many research groups. The primary advantage of pharmacologically conditioning MSCs is that the drugs used to condition the cells have already been assessed for human use. Huang et al. treated MSCs with atorvastatin, a commonly used lipid-lowering medication with pleiotropic functions, and characterized their derivative exosomes (Huang et al. 2020a). In vitro studies revealed enhanced EC survival, migration, and tubulogenesis, while the intramyocardial injection of exosomes significantly reduced infarct size and enhanced cardiomyocyte protection and angiogenesis. It inhibited the expression of TNF-1α and IL-1 in the peri-infarct zone in an experimental model of acute MI. The same researchers have also used a combinatorial approach involving concomitant treatment with atorvastatin-preconditioned MSCs and MSC-derived exosomes in a rodent model of AMI (Huang et al. 2019). Apart from atorvastatin, other pharmacological agents used to prime the cells to modify their exosome payload include oxytocin, pioglitazone, doxorubicin, curcumin, t-PA, etc. (Hu et al. 2021; Han et al. 2021).

As an alternative to manipulating the cells, the noncellular manipulation strategy involves isolating the exosomes and their direct loading with the payload molecules of interest to tailor-made them according to their needs. Akin to cellular manipulation, the noncellular strategy also involves loading exosomes with various biomolecules, proteins, miRs, siRNA, etc. This is achieved by multiple well-established protocols based on sonication, electroporation, transfection, etc., while in some

cases, the desired compounds are mixed and incubated with exosomes at room temperature (Fu et al. 2020).

The other strategy being developed to enhance the therapeutic efficacy of exosomes is to increase the myocardial-directed/specific delivery of exosomes to treat myocardial infarction (Vandergriff et al. 2018). The conjugation of exosomes with cardiac homing peptides enhanced their homing into the ischemic myocardium after intravenous injection while drastically offsetting uptake by nontarget tissue (Vandergriff et al. 2018).

Exosome in Translational Experimental Studies

Based on the encouraging data from the small experimental animal studies, exosomes derived from different stem/progenitor cells are being extensively studied for their therapeutic promise and reparability in translational animal models (Gallet et al. 2017; Adamiak et al. 2018; Maring et al. 2019; Potz et al. 2018). Most of these studies have reported the therapeutic promise of exosomes as good as the cell source of their derivation. Of these cell types, MSCs are the leading source of exosomes used in large animal experimental models (Spannbauer et al. 2020; Hade et al. 2021).

In most experimental studies, as in small experimental animal studies, intramyocardial delivery has been used as the most preferred route of exosome delivery (Gallet et al. 2017; Collantes et al. 2017; Bayardo et al. 2021) (Table 2). However, given the invasiveness of the procedure, it allows a one-time administration of exosomes as an adjunct to routine coronary artery bypass grafting (CABG). As an alternative to a single-dose, one-time administration, researchers are optimizing protocols based on a multidosing strategy scheduled at different time points to achieve a better prognosis (Kisby et al. 2021; Charles et al. 2020). Charles et al. (2020) modified the treatment approach to intravenous bolus delivery of MSC-derived exosomes for 7 consecutive days, twice daily, in a porcine model of myocardial infarction (Charles et al. 2020). The systemic delivery significantly reduced the infacrt size on day 7 and 28 of observation using magnetic resonance imaging (MRI).

Moreover, there was a significant increase in the indices of left ventricle (LV) function and reduced LV-wall thinning compared to the control group of animals. These data clearly show the importance of early and repeated injections of exosomes as a therapeutic approach in terms of safety and efficacy. In a recent study, Zhu et al. used a minimally invasive approach to deliver MSC-derived exosomes in hyaluronic acid gel via intrapericardial injection. The suspension of exosomes in the gel caused the slow release of exosomes from the injection site (Zhu et al. 2021). This approach helped increase the retention of the delivered exosomes, contributed to their slow release, and resulted in higher uptake by the cardiomyocytes. Moreover, hydrogel-based exosome delivery is translatable to the clinics and provides a safe alternative for repeated intravenous injections.

The biodegradable and porous hydrogels also reduce the amount of exosome dose needed to achieve therapeutic benefits due to the reduced loss of the delivered exosomes from the delivery site. Some of the reported hydrogels used for exosomal

Table 2 Summary of translational studies using stem-cell-derived exosomes in large animal models of heart diseases

Author/year	Source of exosomes	Delivery Mode	Model type	Animal	Main findings
Bayardo et al. (2021)	iPSC-derived cardiomyocytes and MSC-derived exosomes	I/M	Acute MI	Porcine	Transendocardial injection of exosomes derived from iPSC-derived cardiomyocytes and MSCs in the preinfarct region – cardiac MRI at 2 and 4 weeks showed significantly improved LVEF and infarct size as compared to the control treated animals. Transcriptome data showed homeostatic preservation of energetics through hibernation of the injured myocardium
Gallet et al. (2017)	CDC-secreted exosomes	I/C or I/M	Acute and chronic MI	Porcine	I/C or I/M delivery of human cardiosphere-derived exosomes (or vehicle as control) 30 min after reperfusion – I/C were ineffective, but I/M delivery preserved LV function. In another set of animals, I/M injection of exosomes 4 weeks after MI led to significantly preserved LVEF and LV volumes as assessed by MRI, decreased scarring, decreased collagne contents and cardiomyocyte hypertrophy with a simultaneous in the blood vessel density
Potz et al. (2018)	Human MSC-derived exosomes	I/M injection	Chronic myocardial ischemia by placement of ameroid ring around LCX	Porcine	Twenty-three Yorkshire pigs divided to receive control treatment ($n = 7$) and exosome treatment ($n = 10$) 2 weeks after the development of the animal model – there was a significant increase in blood vessel density in exosome-treated animals as compared to the control. There was a significant increase in phospho-mitogen-activated protein kinase/mitogen-activated protein kinase ratio and phospho-endothelial nitric oxide synthase/endothelial nitric oxide synthase ratio in the exosome treatment group
Nguyen et al. (2018)	CDC-derived exosomes	I/M injection	LAD occlusion for 150 min, followed by reperfusion for 4 weeks	Porcine	Two groups of control and exosome treatment by intramyocardial injection of placebo or CDC-derived exosomes at 4 weeks after ischemia/reperfusion injury – viability was improved and cardiac function was preserved in the exosome treatment. More importantly, the ultrastructure of the myocardial tissue was significantly preserved in the exosome treatment group

López et al. (2020)	CDC-derived exosomes	Intrapericardial injection	LAD occlusion for 150 min, followed by reperfusion for 4 weeks	Porcine	The animals in the treatment group received intrapericardial injection of exosomes during the early inflammatory phase after myocardial infarction. The authors observed significant changes in biochemical and immunological parameters. There was a significant increase in peripheral blood M2 macrophages. Overall, there was a significant reduction in intraperitoneal and peripheral blood leukocytes. These data show that exosomal treatment has immunomodulatory effects, especially on monocyte polarization. Also, exosomal treatment is proangiogenic
Charles et al. (2020)	MSC-derived exosomes	Twice daily I/V dose for 7 days	MI	Porcine	There was a significant reduction in infarct size on days 7 and 28 of observation using MRI, as well as a significant increase in the indices of LV function and reduced LV-wall thinning as compared to the control group of animals
Zhu et al. (2021)	MSC-derived exosomes in HA gel	Minimally invasive intrapericardial injection	Normal heart	Porcine	The purpose of the study was to ascertain the fate of cells. Sizeable exosome retention in the heart was observed by fluorescent imaging. There was little change in blood chemistry indicators. Confocal microscopic images showed uptake of the exosomes by day 3
Yao et al. (2021)	MSC-derived exosomes	Spray formulation	AMI	Porcine	Designing, fabrication, and testing of a spray of exosomes in biomaterial – the treatment significantly improved cardiac function, reduced cardiac fibrosis, and promoted endogenous angiomyogenesis in the postinjury heart
Monguió-Tortajada et al. (2021)	Cardiac-adipose-tissue-derived or MSC-derived exosomes in peptide hydrogel	Direct placement of cardiac patch with exosomes	MI	Porcine	Treatment with exosome-embedded pericardial scaffold was placed over the infarcted myocardium integrated with the myocardium by day 6 after placement. There was a significant increase in angiogenesis and reduced macrophage T-cell infiltration in the damaged myocardium. The scaffold generated a vascularized niche for the infiltration of resident stem cells to participate in the repair process and modulated short-term post-ischemic inflammation

(continued)

Table 2 (continued)

Author/year	Source of exosomes	Delivery Mode	Model type	Animal	Main findings
Hirai et al. (2020)	Cardiac-adipose-tissue derived exosomes, CDCs, or CDCs pretreated to release exosomes	Intracoronary injection	Porcine model of dilated cardiomyopathy	Porcine	Pretreatment of the cells to block exosome release abrogated the functional recovery of cell-based therapy. Even when exosomes were coinjected with these cells, they failed to recover functional benefits. Improved cardiac indices and reduced cardiac fibrosis were observed in animals treated with naive CDCs. CDC-derived exosomes were rich in proangiogenic and cardioprotective miRs, especially miR-146a-5p, but treatment with isolated exosomes did not recapitulate the beneficial effects. The study led to phase I safety assessment in five pediatric patients to provide a translational framework for the future use of CDCex-derived miRNAs as potential paracrine mediators as a therapeutic strategy

Abbreviations: *AMI* acute myocardial infarction, *BM* bone marrow, *CDCs* cardiosphere-derived cells, *HA* hyaluronic acid, *I/C* intracoronary, *I/M* intramyocardial, *I/V* intravenous, *iPSCs* induced pluripotent stem cells, *LAD* left anterior descending artery, *LCX* left circumflex, *LVEF* Left ventricle ejection fraction, *miR* microRNA, *MI* myocardial infarction, *MSCs* mesenchymal stem cells

preparation include shear-thinning hydrogel (STG), PA-GHRPS, nanocomposite, and hydrogel patches (Chen et al. 2021).

Monguió-Tortajada et al. have used a 3D-scaffold-based delivery of exosomes to create a vascularized niche in the infarcted myocardium to attract resident cells for participation in the repair process (Monguió-Tortajada et al. 2021). The authors of the study used cardiac-adipose-tissue-derived MSCs for the purification of exosomes, which significantly reduced proinflammatory cytokine production (i.e., IFN, TNF-1α, IL12) and promoted angiogenesis. Interestingly, within 6 days after implantation, the engineered scaffold carrying exosomes was found to integrate with the recipient infarcted myocardium efficiently.

Recent advancement in the fast-developing exosome-based therapeutic approach is the site-directed delivery of exosomes, avoiding their spillover to the nontargeted tissues and organs (Barjesteh et al. 2021). The development of inorganic nanoparticle-loaded exosomes is gaining popularity in this regard. Lee et al. have recently reported using exosome-mimetic nanovesicle derived from iron oxide nanoparticle-loaded MSCs (IONP-MSCs) (Lee et al. 2020). These iron-oxide-containing nanovesicles were magnetically guided to the infarcted porcine heart muscle, which significantly reduced the duration of the inflammatory phase, reduced apoptosis and fibrosis, and increased angiogenesis. Overall, the authors reported a significantly improved functional recovery of the infarcted heart and proposed the potential feasibility and target specificity of the treatment approach, besides alleviating the need for large quantities of exosomes. Moreover, with the encouraging data emanating from the translational studies, GMP compliance is the need of the hour to forward an exosome-based treatment approach to the clinics for therapeutic applications. Hence, attempts are underway to optimize protocols for the large-scale clinical-grade production of exosome preparation for human use in clinical settings (Andriolo et al. 2018).

Exosome in Clinical Studies

Despite encouraging translational data from large animal studies and their advantages and superiority (i.e., safety and feasibility) over mother cells from which have been derived, little progress has been made in their use for myocardial repair and regeneration in clinical settings. As of the writing of this chapter (October 30, 2021), there are **112 clinical trials** listed on www.clinicaltrials.org using exosomes. These trials are primarily about noncardiac conditions, including diabetes types I and II, stroke, chronic kidney disease, macular degeneration, cancer and cancer-associated conditions, COVID-19, acute respiratory distress syndrome (ARDS), etc. (Table 3). Moreover, some of the studies are intended to ascertain the diagnostic potential of the exosomes rather than the assessment of their therapeutic value. It is pertinent to mention here that though exosomes have tremendous therapeutic potential, it would be important that exosome preparations are clinical grade to ensure their safety for human applications.

More recently, the FDA has cleared three IND (Investigational New Drug) applications based on PEP™ (Purified Exosome Preparations) for three different conditions, including wound healing (Phase 1b/2a study; ClinicalTrials.gov

Table 3 Clinical trials assessing the therapeutic and diagnostic significance of exosomes for different pathological conditions. (Data have been derived from ClinicalTrials.gov)

NCT identifier	Study title	Disease	Method of intervention	Institution and location of the trial
Therapeutic applications of exosomes in clinical trials				
ClinicalTrials.gov identifier: NCT03478410	Role of exosomes derived from epicardial fat in atrial fibrillation	Atrial fibrillation	Procedure: epicardial fat biopsy	Sheba Medical Center, Ramat Gan, Israel
ClinicalTrials.gov identifier: NCT03384433	Allogenic MSC-derived exosome in patients with acute ischemic stroke	Cerebrovascular disorders	Biological: exosome	Shahid Beheshti University of Medical Sciences, Tehran, Iran
ClinicalTrials.gov identifier: NCT04798716	A pilot clinical study on the inhalation of MSC exosomes treating severe novel coronavirus pneumonia	Coronavirus	Biological: MSC-derived exosomes	Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
ClinicalTrials.gov identifier: NCT04270006	Evaluation of adipose-derived stem cell exosomes for the treatment of periodontitis	Periodontitis	Biological: adipose-derived stem cell exosomes	Beni-Suef University, Banī Suwayf, Egypt; Cairo University, Egypt
ClinicalTrials.gov identifier: NCT02565264	Effect of plasma-derived exosomes on cutaneous wound healing	Ulcer	Other: plasma-derived exosomes	Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
ClinicalTrials.gov identifier: NCT04849429	Intradiscal injection of platelet-rich plasma (PRP) enriched with exosomes in chronic low back pain	Chronic low back pain, degenerative disk disease	Biological: platelet-rich plasma (PRP) with exosomes	Anupam Hospital, Uttarakhand, India; Mother Cell Spinal Injury and Stem Cell Research, Anupam Hospital, India
ClinicalTrials.gov identifier: NCT05060107	Intra-articular injection of MSC-derived exosomes in knee osteoarthritis (ExoOA-1)	Osteoarthritis, knee	Biological: exosomes (EVs)	
ClinicalTrials.gov identifier: NCT04213248	Effect of UMSC-derived exosomes on dry eye in patients with cGVHD	Dry eye	Drug: umbilical MSC- derived exosomes	Zhongshan Ophthalmic Center, Guangzhou, Guangdong, China

ClinicalTrials.gov identifier: NCT02138331	Effect of microvesicle and exosome therapy on β-cell mass in T1DM	T1DM	Biological: MSC exosomes	Sahel Teaching Hospital, Sahel, Cairo, Egypt
ClinicalTrials.gov identifier: NCT03437759	MSC-Exos promoting healing of MHs	MHs	Biological: MSC-derived exosomes (MSC-Exo)	Tianjin Medical University Hospital, Tianjin, China
ClinicalTrials.gov identifier: NCT04388982	The safety and efficacy evaluation of allogenic adipose MSC-Exos in patients with Alzheimer's disease	Alzheimer's disease	Biological: low-, mild-, and high-dosage MSC-Exos administrated for nasal drip	Ruijin Hospital affiliated with Shanghai Jiaotong University School of Medicine, Shanghai, China
ClinicalTrials.gov identifier: NCT04602442	Safety and efficiency of the method of exosome inhalation in COVID-19-associated pneumonia	• Covid19 • SARS-CoV-2 PNEUMONIA • COVID-19	• Drug: EXO 1 inhalation • Drug: EXO 2 inhalation • Drug: placebo inhalation	Medical Centre Dynasty Samara, Russian Federation
ClinicalTrials.gov identifier: NCT04154332	Exosome cargo from preeclampsia patients	Preeclampsia	Other: sample collection	University of Alabama at Birmingham, Birmingham, Alabama, United States
ClinicalTrials.gov identifier: NCT04879810	Plant exosomes +/− curcumin to abrogate symptoms of inflammatory bowel disease	Irritable bowel disease	Procedure: sigmoidoscopy and biopsy, blood work	University of Louisville, Louisville, Kentucky, United States
Diagnostic applications of exosomes in clinical trials				
ClinicalTrials.gov identifier: NCT04127591	Differential expression and analysis of peripheral plasma exosome miRNA in patients with MI	MI	Other: exosome	Ethics Committee of Xinhua Hospital, Shanghai, China
ClinicalTrials.gov identifier: NCT04182893	Clinical study of ctDNA and exosome-combined detection to identify benign and malignant pulmonary nodules	Pulmonary nodules	Diagnostic test: ctDNA and exosome-combined detection	Shanghai Chest Hospital, Shanghai, China
ClinicalTrials.gov identifier: NCT04894695	Urine exosomes to identify biomarkers for LN	Lupus nephritis	Diagnostic test: biomarker detection in different groups	Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

(continued)

Table 3 (continued)

NCT identifier	Study title	Disease	Method of intervention	Institution and location of the trial
ClinicalTrials.gov identifier: NCT04529915	Multicenter clinical research for the early diagnosis of lung cancer using blood-plasma-derived exosome	Lung cancer	Diagnostic test: exosome sampling	Korea University Guro Hospital, Guro-gu, Seoul, Republic of Korea
ClinicalTrials.gov identifier: NCT04053855	Evaluation of urinary exosome presence from clear cell renal cell carcinoma	Clear cell renal cell carcinoma	Biological: urinary sample	Chu Saint-Etienne, Saint-Étienne, France
ClinicalTrials.gov identifier: NCT03800121	Study of exosomes in monitoring patients with sarcoma (EXOSARC)	Sarcoma	Biological: blood samples	CHU de Besançon, Besançon, France, Centre Georges François Leclerc, Dijon, France
ClinicalTrials.gov identifier: NCT05058768	Omics sequencing of exosomes in body fluids of patients with acute lung injury	Acute lung injury	Diagnostic test: the lung injury, causes and extrapulmonary factors	Nanfang Hospital, Southern Medical University, Guangzhou, China
ClinicalTrials.gov identifier: NCT03830619	Serum exosomal long noncoding RNAs as potential biomarkers for lung cancer diagnosis	Lung cancer	Diagnostic test: collecting samples	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hub
ClinicalTrials.gov identifier: NCT03874559	Exosomes in rectal cancer	Rectal cancer	Diagnostic test: blood draw	University of Kansas Medical Center/ Cancer Center, Kansas City, Kansas, USA

Abbreviations: *cGVHD* chronic graft versus host disease, *IBD* irritable bowel syndrome, *MHs* macular holes, *MSCs* mesenchymal stem cells, *PRP* platelet-rich plasma, *TD1* type I diabetes mellitus

identifier: NCT04664738), fistulizing Crohn's disease, and acute myocardial infarction (AMI). For AMI, a phase Ib/2a entitled "Safety Evaluation of Intracoronary Infusion of Extracellular Vesicles in Patients With AMI" has been initiated (ClinicalTrials.gov identifier: NCT04327635). Led by the principal investigator Dr. Guy Reeder at Mayo Clinic, the interventional study is currently recruiting patients (21–85 years of age) to research the dose-limiting toxicities and maximum tolerated dose of a single dose of PEP™ (during PCI or AMI) at escalating concentrations of EVs with well-defined exclusion and inclusion criteria expected to start from December 2021. The study will involve the evaluation of three different doses of PEP™ (5%, 10%, and 20%) to be administered via intracoronary infusion within 20 min of stent placement during percutaneous coronary intervention (PCI) at least 4 h (but no more than 12 h) after the onset of heart attack symptoms. The patients would be followed up for 1 year for evaluation.

Another clinical trial registered on ClinicalTrials.gov involving patients with AMI is for the diagnostic application of exosomes in the peripheral circulation (ClinicalTrials.gov identifier: NCT04127591). The study entitled "Differential Expression and Analysis of Peripheral Plasma Exosome miRNA in Patients with Myocardial Infarction" has been designed to study the possible changes in the miR expression profile in peripheral plasma exosomes during an infarction episode. Led by Dr. He from Xinhua Hospital, Shangai Jiao Tong University, China, the study was expected to be completed by December 2021.

Some of the clinical trials for the assessment of the therapeutic efficacy of exosome preparations have been summarized in Table 3 with a summary of the salient results of these studies.

Challenges and Future Perspective

The transfer of cell-free therapy based on a stem-cell-derived exosome approach from bench to bedside as a routine therapeutic and diagnostic modality has immense potential. Unlike their mother cells, although exosomes lack proliferation and differentiation potential, they can promote the mobilization and homing of intrinsic/resident stem/progenitor cells to participate in reparative and regenerative processes. As is evident from the published data, the theranostic assessment of exosomes has reached the clinical phase based on safety and efficacy data from the translational studies (Table 3). However, it still requires optimization, and standardization of the protocols that encompass their isolation, characterization and large scale production in accordance with the GMP requirements to the biological and functional characterization, ensure off-the-shelf availability (i.e., storage under optimal conditions to sustain their efficacy), and safe and efficient use in the humans for theranostic applications. Table 4 summarizes some of the challenges that need to be encountered and overcome to streamline their progress to the clinics.

Table 4 Challenges impeding the progress of exosomes for theranostic applications in the clinics

Pharmaceutical challenges
• Optimal source for exosome isolation, i.e., stem/progenitor cells, peripheral blood, plant, food sources, etc.
• Labor-intensive and time-consuming isolation and purification protocols
• Flawed production and quality control of GMP-grade exosome preparations on a large scale
• Variation in size, shape, and inherent payload
• Maintenance of uniform loading efficiency with the required cargo
• Standardized biophysical, biochemical, and biological characterization of exosome preparations
• Lack of optimized protocols for long-term storage and stability (i.e., time, temperature, freezing, thawing, reconstitution) to ensure off-the-shelf availability
Preadministration challenges
• Pharmacodynamical features of exosome preparation
• Optimal dosage form for efficient delivery
• Mode of delivery
• Route of administration
• Time of administration
• Dose of administration
• Single- or multi-dose administration
• Site-directed or tissue-targeted administration
• Off-targeted distribution of delivered exosomes
Postadministration challenges
• Lack of optimization of pharmacokinetic features
• Preferential uptake and accumulation in the spleen and liver
• Biodistribution and bioavailability features
• Lack of standardized release profile of the exosome payload
• Biological half-life
• Long-term safety and efficacy concerns
• Toxicological features of exosome preparation
• Postdelivery visualization and fate determination of delivered exosomes

Cross-References

► Extracellular Vesicles-Based Cell-Free Therapy for Liver Regeneration
► Mesenchymal Stem Cells for Cardiac Repair

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Current Trends and Future Outlooks of Dental Stem-Cell-Derived Secretome/ Conditioned Medium in Regenerative Medicine

35

Israa Ahmed Radwan, Dina Rady, Sara El Moshy, Marwa M. S. Abbass, Khadiga Mostafa Sadek, Aiah A. El-Rashidy, Azza Ezz El-Arab, and Karim M. Fawzy El-Sayed

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I. A. Radwan · D. Rady · S. El Moshy · M. M. S. Abbass
Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt
e-mail: esraa.ahmed@dentistry.cu.edu.eg; dina.radi@dentistry.cu.edu.eg; sarah.mahmoud@dentistry.cu.edu.eg; marwa.magdy@dentistry.cu.edu.eg

K. M. Sadek · A. A. El-Rashidy
Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Biomaterials Department, Faculty Dentistry, Cairo University, Cairo, Egypt
e-mail: Khadiga.sadek@dentistry.cu.edu.eg; aiah.abdelwahab@dentistry.cu.edu.eg

A. Ezz El-Arab
Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt
e-mail: azza.ezzelarab@dentistry.cu.edu.eg

K. M. Fawzy El-Sayed (✉)
Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian Albrechts University, Kiel, Germany

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Abstract

Constructing biological substitutes that mimic the structure, architecture, and function of different tissues and organs is the ultimate goal of regenerative medicine. Adult mesenchymal stem/progenitor cells (MSCs) are considered the most widely researched cells in regenerative applications, yet several obstacles that challenge the safe and effective clinical translation of MSC-based therapies still exist. MSCs could partially exert their reparative and regenerative impact through a paracrine effect, mediated by the release of bioactive and trophic factors known as secretome, rather than the actual presence of the engrafted cells in the target site. In addition, MSCs have shown the ability to secrete these various bioactive molecules in their surrounding media (the conditioned media (CM)). MSC-secretome/CM is a set of proteins, lipids, nucleic acids, and trophic factors such as cytokines, chemokines, hormones, growth factors, and extracellular vesicles (EVs). Compared to nondental MSC secretome, dental MSC secretome/CM revealed a higher expression of proliferation-related, metabolic, transcriptional proteins and chemokines, as well as neurotrophins. Dental MSC secretome/CM exhibited experimentally tremendous biological effects, including immunomodulatory, anti-inflammatory, neuroprotective, osteogenic, angiogenic, and antiapoptotic effects, as well as the modulation of

oxidative stresses. These aforementioned biological effects greatly explain the increasing interest in dental MSC secretome/CM as an acellular regenerative strategy for the treatment of various clinical diseases/injuries while alleviating the limitations and safety concerns associated with MSC-based therapies.

Keywords

Cell-free therapy · Conditioned medium · Dental stem cells · Exosomes · Extracellular vesicles

Abbreviations

3D	Three-dimensional
ABMSCs	Alveolar bone proper-derived stem/progenitor cells
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ASCs	Adipose stem/progenitor cells
bFGF	Basic fibroblast growth factor
BMMSCs	Bone-marrow-derived mesenchymal stem/progenitor cells
BMP	Bone morphogenetic protein
BNDF	Brain-derived neurotrophic factor
BSP	Bone sialoprotein
CCK-8	Cell count kit-8
c-JUN/JNK	c-JUN/N-terminal kinase
CM	Conditioned medium
CSF	Colony-stimulating factor
CXCL	CXC motif ligand
DFSCs	Dental follicle stem/progenitor cells
DMSCs	Dental mesenchymal stem/progenitor cells
DPSCs	Dental pulp stem/progenitor cells
DSPP	Dentin sialophosphoprotein
EAE	Encephalomyelitis
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMVs	Exosomes/Matrix vesicles
EVs	Extracellular vesicles
EXs	Exosomes
FGF	Fibroblast growth factor
FGF-R	Fibroblast growth factor-receptor
FLT-3	Fms-like tyrosine kinase receptor-3
GCSF	Granulocyte-colony-stimulating factor
GDNF	Glial-cell-line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMSCs	Gingival mesenchymal stem cells

HEGF	Heparin-binding epidermal growth factor
HFSCs	Hair follicle stem cells-CM
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen-DR isotype
HUVECs	Human umbilical vein endothelial cell culture
IFNγ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukins
iNOS	Inducible nitric-oxide synthase
KGF	Keratinocyte growth factor
LF	Liver failure
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MS	Multiple sclerosis
MSCs	Mesenchymal stem/progenitor cells
MVs	Microvesicles
NF-Kb	Nuclear factor-kappa B
NGF	Neural growth factor
NT-3	Neurotrophin-3
OCN	Osteocalcin
Oct	Octamer-binding transcription factor
OSM	Oncostatin M
PCNA	Proliferating cell nuclear antigen
PDEGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem/progenitor cells
PEI	Polyethyleneimine
PGE2	Prostaglandin E2
PLA	Poly-(lactide)
RUNX2	Runt-related transcription factor 2
SCAP	Stem/progenitor cells from apical dental papilla
SCF	Stem cell factor
SDF	Stromal-cell-derived factor
SHED	Human shed deciduous teeth
SOD	Superoxide dismutase
SOX-2	Sex-determining region Y (SRY)-box 2
sSiglec-9	Soluble sialic-acid-binding Ig-like lectin-9
TFIP11	Tuftelin-interacting protein
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAP	Tartrate-resistant acid phosphatase

TUFT1	Tuftelin 1
VEGF	Vascular endothelial growth factor

Introduction

The restoration, regeneration, or even repair of damaged tissues and organs is the center of interest of many researchers, aiming to improve the quality of life of many patients in need. Regenerative medicine represents a promising treatment modality that, through employing tissue engineering approaches, can enhance and guide the restoration, maintenance, and improvement of different organs and tissue functions (Atala 2008). Regenerative medicine is a multidisciplinary branch of medicine that aims at constructing biological substitutes that can mimic the patient's organs and tissues, thus offering a promising therapeutic modality for several disorders and diseases (Berthiaume et al. 2011; Stoltz et al. 2015). The tissue engineering approach combines biocompatible scaffolds, cells, and biologically active molecules into functional tissues (Berthiaume et al. 2011; Gao and Cui 2016; Guan et al. 2017), while regenerative medicine, being a broader field, includes tissue engineering approaches and research on self-healing, where the body can use its endogenous tissue formation ability, which could occur secondary to induction by foreign biological materials, to form new cells and regenerate tissues and organs (Katari et al. 2015).

Biocompatible scaffolds employed in tissue engineering are commonly fabricated from bioceramics, polymers, or their composites. Bioceramic and bioactive glass scaffolds and their composites are widely employed (Bose et al. 2012; Pilia et al. 2013; Roseti et al. 2017; Chocholata et al. 2019; Kerativitayanan et al. 2017). In addition, various polymeric scaffolds are fabricated from natural, synthetic, or hybrid polymers in addition to elastic polymer networks such as hydrogels (Rezwan et al. 2006; Pina et al. 2015; Qu et al. 2019). Scaffold biomaterials are often combined with bioactive molecules and/or growth factors that can home, stimulate, and promote the differentiation of tissue-resident stem/progenitor cells (Gomes and Reis 2004; Mallick et al. 2015; Chocholata et al. 2019). Decellularization is an innovative scaffold fabrication technique, where the extracellular matrix (ECM) with its organization, vascular network, and architecture are preserved. Decellularization provides a three-dimensional (3D) cell-free scaffold, ready to be seeded with the desired cell type for specific tissue engineering applications (Paduano et al. 2017; Taylor et al. 2017); such 3D scaffold influences cellular behavior and differentiation potential through its structure and biological signaling (He and Callanan 2013). The decellularization process could be achieved by various methods, which employ enzymes, detergents, and salts combined with specific physical stimuli (Scarritt et al. 2015).

Tissue engineering employs several cell types, benefiting from their remarkable properties in each intended application; cells employed include primarily embryonic stem cells, adult stem/progenitor cells, in addition to induced pluripotent stem cells (Egusa et al. 2012, Shah et al. 2016). Adult mesenchymal stem/progenitor cells

(MSCs) are considered the most widely investigated cell population in tissue engineering applications based on their multipotency and differentiation potential into an array of mesodermal origin, upon proper stimulation, as well as its self-renewal capabilities. MSCs reside in many locations in adult tissues, including bone marrow, umbilical cord, synovial fluid, and adipose tissues (Pires et al. 2016; Hsiao et al. 2012; Jones et al. 2004).

The cellular transplantation of MSCs has been proposed as a treatment modality for functional tissue regeneration and for treating various autoimmune and chronic inflammatory diseases. Unfortunately, the clinical translation of stem-cell-based therapies faces various serious ethical and safety issues (Volarevic et al. 2018). Based on the well-documented paracrine hypothesis (Haider and Aziz 2017), a more promising alternative is the utilization of MSCs-conditioned media (CM), as recently MSCs have been characterized by their ability to secrete various bioactive molecules into their surrounding media (Lei and Haider 2017). Such secreted bioactive molecules, known as secretome, can be easily collected and have been remarkable in the enhancement of mesenchymal tissue regeneration (Caplan 2007; Cicconetti et al. 2007). This book chapter aims to review the efficacy of secretome derived from different populations of dental mesenchymal stem/progenitor cells (DMSCs) in the treatment of various diseases and the regeneration of different tissues, emphasizing and displaying the involved content of bioactive molecules.

Dental Stem/Progenitor Cells (DMSCs)

DMSCs are multipotent adult MSCs derived from the ectomesenchymal neural crest cells (Fawzy El-Sayed et al. 2013a; Fawzy El-Sayed et al. 2013b). DMSCs include stem/progenitor cells extracted from pulpal tissues of human shed deciduous teeth (SHED) (Miura et al. 2003; Stanko et al. 2018), dental pulp stem/progenitor cells (DPSCs) isolated from dental pulpal tissues of permanent teeth (Gronthos et al. 2000), stem/progenitor cells isolated from apical papilla (SCAP) at the apices of immature permanent teeth (Jo et al. 2007; Sonoyama et al. 2006), tooth germ progenitor cells isolated from late bell stage third molar tooth germs (Ikeda et al. 2008), dental follicle stem/progenitor cells (DFSCs) isolated from dental follicle surrounding the third molar (Morsczeck et al. 2005), periodontal ligament stem/progenitor cells (PDLSCs) isolated from the periodontal ligament (Seo et al. 2004; Jo et al. 2007), alveolar bone proper-derived stem/progenitor cells (ABMSCs) (Fawzy El-Sayed et al. 2012a, 2014, 2017), and gingival stem/progenitor cells (GMSCs) isolated from gingival tissues (Palmer and Lubbock 1995; Fawzy-El-Sayed et al. 2016, 2019b; Fawzy El-Sayed and Dorfer 2016; El-Sayed et al. 2015). Stem/progenitor cells were also isolated from inflamed pulp (Alongi et al. 2010; Malekfar et al. 2016) and periapical cysts (Marrelli et al. 2013; Tatullo et al. 2017).

DMSCs are characterized by their multidifferentiation potential into multiple cell lineages, self-renewal ability, potent regenerative potentials, and immunomodulatory properties (Cordeiro et al. 2008; Dominici et al. 2006; Huang et al. 2010; Leucht et al. 2008; Liu et al. 2015; Nakashima and Iohara 2014; Park et al. 2010).

In addition to being easily acquired with minimally invasive procedures (Egusa et al. 2012), they were reported to have improved regenerative potential when compared to MSCs derived from other sources. SHED (Isobe et al. 2016), DPSCs (Murakami et al. 2015; Kumar et al. 2017a, b, 2018; Mead et al. 2014; Davies et al. 2015; Isobe et al. 2016), SCAP (Kumar et al. 2017a, b, 2018), and DFSCs (Kumar et al. 2017a, b, 2018) were shown to exhibit higher neurogenic (Isobe et al. 2016; Kumar et al. 2017a), angiogenic (Murakami et al. 2015; Song et al. 2017), osteogenic (Kumar et al. 2018; Davies et al. 2015), hepatogenic (Kumar et al. 2017b), antiapoptotic (Murakami et al. 2015), and pulpal tissue (Murakami et al. 2015) regenerative potential and higher proliferative rates (Abdullah et al. 2014; Wang et al. 2012) in comparison to adipose stem/progenitor cells (ASCs) (Mead et al. 2014; Davies et al. 2015; Murakami et al. 2015) or bone-marrow-derived mesenchymal stem/progenitor cells (BMMSCs) (Isobe et al. 2016; Murakami et al. 2015; Kumar et al. 2017a, b, 2018; Mead et al. 2014; Davies et al. 2015).

DMSCs express common MSC surface markers, including CD105, CD73, and CD90, with a lack of expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and human leukocyte antigen-DR isotype (HLA-DR) (Huang et al. 2009).

Stem/Progenitor Cells' Secretome/Conditioned Medium

Besides the direct cellular activity of stem/progenitor cells following their transplantation, they can exert an indirect paracrine effect to enhance tissue repair and regeneration **(**Fig. 1**)** (Ankrum and Karp 2010; Baglio et al. 2012; Maguire 2013; Wollert and Drexler 2010). Such paracrine effect is promoted through the secretion of secretome into their surrounding tissues, which exerts an immunomodulatory effect that supports and enhances tissue regeneration and homeostasis, besides enhancing cell migration and proliferation (Bai et al. 2016; Baraniak and McDevitt 2010; Beer et al. 2017; Katagiri et al. 2013; Madrigal et al. 2014; Li et al. 2014; Ranganath et al. 2012). Based on such effects, cell-free therapy, employing stem/progenitor cells' secretome, has emerged as an alternative treatment modality to cell-based therapies (Baglio et al. 2012; Ciapetti et al. 2012; Maguire 2013).

As discussed earlier, the secretome is an array of bioactive molecules secreted from living MSCs or shed from their surface into the surrounding environment (Beer et al. 2017).

This can be replicated in vitro through the stimulation of stem/progenitor cells to release secretome and trophic factors into the culture media, producing the stem/progenitor cells' CM (Baraniak and McDevitt 2010; Phelps et al. 2018). Stem/progenitor-cell-derived secretome has shown several advantages over cell-based therapy, which involves the ease of secretome preservation, sterilization, packaging, and storage for long time durations without the risk of losing its properties. Secretome dosage can be precisely calculated and the production of large quantities can be readily achieved using cell lines, which save time and cost while avoiding any invasive procedures to the patient (Bermudez et al. 2015, 2016; Osugi et al. 2012; Vizoso et al. 2017).

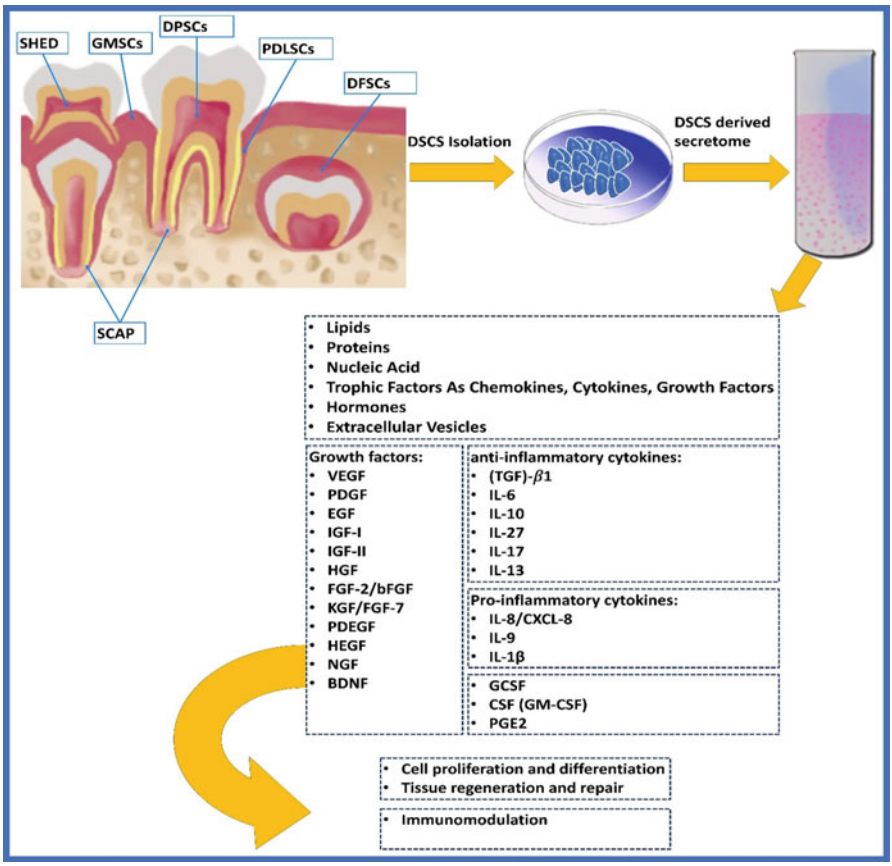

Fig. 1 Illustrative diagram showing various bioactive molecules detected in secretome derived from dental mesenchymal stem cells

Stem/progenitor cells' secretome is composed of proteins, nucleic acid, lipids, extracellular vesicles (EVs), and trophic factors such as chemokines, cytokines, growth factors, and hormones (Ranganath et al. 2012), and its content varies according to the anatomic location of the stem/progenitor cells (Assoni et al. 2017). Stem/progenitor cells' secretome was demonstrated to harbor an array of growth/differentiation factors, including platelet-derived growth factor (PDGF); vascular endothelial growth factor (VEGF); platelet-derived endothelial cell growth factor (PDEGF); insulin-like growth factors I, II (IGF-I, IGF-II); epidermal growth factor (EGF); keratinocyte growth factor/fibroblast growth factor-7 (KGF/FGF-7); fibroblast growth factor 2/basic fibroblast growth factor (FGF-2/bFGF); hepatocyte growth factor (HGF); heparin-binding epidermal growth factor (HEGF); neural growth factor (NGF); and brain-derived neurotrophic factor (BDNF) (Pawitan 2014). In addition to its content of anti-inflammatory cytokines, including transforming growth factor (TGF)-β1 and interleukins (ILs), including IL-6, IL-10, IL-27,

IL-17, and IL-13, and proinflammatory cytokines, including IL-8/CXC motif ligand (CXCL)-8, IL-9, and IL-1β. Granulocyte-colony-stimulating factor (GCSF), granulocyte-macrophage CSF (GM-CSF), and prostaglandin E2 (PGE2) (Pawitan 2014).

Extracellular Vesicles (EVs)

EVs include exosomes (EXs) (40–100 nm), microvesicles (MVs) (100–1000 nm), and apoptotic bodies (1–5 um), and their content depends on the surrounding environment and may change upon cell stimulation. They are secreted by many cell types, including stem/progenitor cells, and they can be isolated from body fluids, like serum, cerebrospinal fluids, and urine (Beer et al. 2017; Lee et al. 2012; Raposo and Stoorvogel 2013; Skog et al. 2008). Upon reaching their target sites, EVs interact with and attach to the target cell surface, then they either remain attached or become internalized via endocytosis or membrane fusion to the target cell plasma membrane to release their content into the recipient cells or become detached from the cell surface following the completion of their action (Kim et al. 2013; Raposo and Stoorvogel 2013).

Exosomes (EXs) and Microvesicles (MVs)

EXs and MVs are membrane-bound particles, secreted for normal homeostasis by many cell types, and their secretion increases upon stimulation (Kim et al. 2013; Valadi et al. 2007). EXs and MVs differ in their origin (biogenesis) and physical characteristics, such as size and surface markers (Lee et al. 2012; Mathivanan et al. 2010; Ratajczak et al. 2006). Their content varies according to the producing cells, comprising proteins and lipids, along with protein-coding messenger ribonucleic acids (mRNAs) and noncoding microRNAs (Gyorgy et al. 2011; Ratajczak et al. 2006; Skog et al. 2008; Valadi et al. 2007; Haider and Aramini 2020). EXs and MVs are essential for intercellular communication, with both exerting paracrine and endocrine actions (Kim et al. 2013). EXs and MVs act as vehicles for the transport of bioactive molecules, such as cytokines and growth factors, from producing cells to adjacent or distant target cells via circulation (Kim et al. 2013; Raposo and Stoorvogel 2013; Valadi et al. 2007). They can also modify target cells' gene expression or protein synthesis through delivering RNA (Nakamura et al. 2015; Tomasoni et al. 2013).

EXs are homogenous and smaller in size than MVs, with a diameter ranging from 40 to 100 nm. They originate in multivesicular bodies, which are discharged through exocytosis via fusion with the cell membrane (Lee et al. 2012; Tkach and Thery 2016). Multivesicular bodies are late endosomes that are formed by the maturation of early endosomes; early endosomes are formed following the fusion of endocytotic vesicles (Pant et al. 2012). EXs are rich in annexins, tetraspanins (CD63, CD81, and CD9), and heat-shock proteins (such as Hsp60, Hsp70, and Hsp90), which are usually used for their identification (Biancone et al. 2012). On the other hand,

MVs (also termed ectosomes) are heterogeneous and larger in size than EXs, with a diameter ranging between 100 and 1000 nm. MVs are produced through direct budding from the cell plasma membrane, and their surface markers originate from the producing cells (Mathivanan et al. 2010; Vishnubhatla et al. 2014). MVs are rich in proteins, lipids, as well as mRNAs and microRNAs (Collino et al. 2010).

Comparison Between Secretome/CM Derived from DMSCs and MSCs from Other Tissue Sources

Proteomic analysis identified a total of 1533 proteins in the CM derived from BMMSCs, ASCs, and DPSCs. Nine hundred ninety-nine proteins were common among all three cell sources, which included 124 proteins identified as secreted extracellular proteins. These secreted extracellular proteins are proposed to modulate MSCs' regenerative potential, such as inflammatory response, angiogenesis, cell migration, ossification, and organ survival. Proteins isolated from BMMSCs-CM were similar to those isolated from ASCs-CM but did not resemble proteins isolated from DPSCs-CM (Tachida et al. 2015). Proteins responsible for immunomodulation, angiogenesis, neuroprotection, chemotaxis, antiapoptosis, and extracellular matrix formation were expressed in both SCAP-CM and BMMSCs-CM. However, there was a significant difference in the expression levels of 151 proteins between the two cell sources, where SCAP-CM showed higher expression of proteins associated with metabolic processes and transcription, along with chemokines and neurotrophins, while expressing lower levels of proteins related to immunomodulation, angiogenesis, adhesion, and extracellular matrix proteins (Yu et al. 2016). One hundred seventy-four cytokines were commonly expressed in SCAP-CM, DFSCs-CM, and DPSCs-CM. Notably, a significantly higher expression of 23 cytokines related to odontoblast differentiation and proinflammatory and anti-inflammatory cytokines was evident in DPSCs-CM, while three cytokines responsible for cellular proliferation were significantly highly expressed in DFSCs-CM and SCAP-CM (Joo et al. 2018).

DPSCs-CM showed higher angiogenic, antiapoptotic, and neurite outgrowth; migration activity (Ishizaka et al. 2013; Murakami et al. 2015); and immunomodulation in vitro when compared to BMMSCs-CM, as well as higher vasculogenesis in vivo (Ishizaka et al. 2013). In addition, DPSCs-CM revealed increased migration and angiogenic activity and antiapoptotic effect on mouse embryonic muscle myoblast cells (C2C12); in vitro, this could be based on its high content of monocyte chemoattractant protein-1 (MCP-1) and CXCL14 (Hayashi et al. 2015). A superior nerve regenerative potential was related to DMSCs-CM derived from DPSCs, SCAP, and DFSCs when compared to BMMSCs-CM, evident by the significantly higher colony formation and neurite extension, indicating an enhanced neural differentiation and maturation associated with DMSCs-CM, in comparison to BMMSCs-CM. Such superior neuroregenerative potential can be explained by the significantly higher expression of BDNF, neurotrophin-3 (NT-3) in DMSCs-CM derived from all three cell sources, and the significantly higher expression of NGF in

SCAP-CM and DFSCs-CM when compared to BMMSCs-CM. Higher levels of interferon gamma (IFNγ), TGF-β, and GCSF were also expressed in DPSCs-CM when compared to BMMSCs-CM (Kumar et al. 2017a). Similar results were notable upon comparing DPSCs-CM with BMMSCs-CM and ASCs-CM (Mead et al. 2014).

Secretome/Conditioned Medium (SHED-CM) Derived from Stem Cells from Exfoliated Human Deciduous Teeth

SHED obtained from the pulpal tissues of deciduous teeth exhibits a higher proliferation rate as compared to DPSCs and BMMSCs. Microarray analysis revealed that SHED had higher expression levels of FGF, TGF, connective tissue growth factor, NGF, and bone morphogenetic protein (BMP)-1 (Nakamura et al. 2009). Genes encoding for extracellular, cell surface molecules; cell proliferation; and embryonic tissue development are highly expressed by SHED. Moreover, SHED neurogenic potential might be related to their neural crest embryonic origin. SHED expressed neural cell lineage markers, including nestin, doublecortin, β-tubulin III, NeuN, glial fibrillary acidic protein (GFAP), S-100, A2B5, and 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (Sakai et al. 2012). Moreover, SHED releases an array of secretomes with multiple biological therapeutic activities.

SHED-CM in Treating Cardiopulmonary Injuries

In vitro, SHED-CM promoted the differentiation of mouse bone-marrow-derived macrophages into M2 macrophages, which expressed Arginase1, Ym-1, and CD206. These findings were further verified in vivo (Hirata et al. 2016; Matsushita et al. 2017; Wakayama et al. 2015) in a bleomycin-induced acute lung injury mice model; the intravenous administration of SHED-CM reduced lung fibrosis and enhanced survival rates. These therapeutic effects could be a result of the reduced expression of proinflammatory cytokines and fibrotic markers, such as α-smooth muscle actin, thus altering proinflammatory M1 into an anti-inflammatory M2 phenotype (Wakayama et al. 2015; Matsubara et al. 2015; Yamagata et al. 2013). Moreover, SHED-CM revealed cardioprotective benefits in ischemic heart diseases by at least two mechanisms, including the suppression of inflammatory responses in myocardial cells and the reduction of cardiomyocyte death. Interestingly, SHED-CM showed superior effects compared to those of BMMSC-CM and ASC-CM; this may be due to the significantly higher expression of HGF in SHED-CM as compared to the other two cell sources (Yamaguchi et al. 2015).

SHED-CM in Treating Hepatic Disorders

Single intravenous administration of SHED-CM in a liver failure (LF) mice model revealed a significant therapeutic effect that was not detected in fibroblast-CM

(Hirata et al. 2016; Matsushita et al. 2017). In vitro, SHED-CM suppressed chronic inflammation and induced tissue repair, where tumor necrosis factor-α (TNF-α), IL-1β, and inducible nitric oxide synthase (iNOS) were strongly suppressed. In addition, SHED-CM induced hepatic stellate cells' apoptosis and protected hepatocytes from apoptosis by suppressing carbon-tetrachloride-induced apoptosis in hepatocytes (Hirata et al. 2016). SHED-CM attenuated the LF-induced pro-inflammatory response and generated an anti-inflammatory environment, where SHED-CM stimulated M2 cell markers (CD206 and Arginase1), anti-inflammatory cytokines (IL-10 and TGF-β1), angiogenic factor (VEGF) and antiapoptotic factor (stem cell factor (SCF) and IGF-1) expression, and hepatocyte proliferation. Furthermore, SHED upregulated the expressions of lysophosphatidylcholine activation genes, including HGF, TNF-related weak inducer of apoptosis (TWEAK), FGF-7, and Wnt3a (Matsushita et al. 2017). These data propose that the active biomolecules within the SHED-CM and the endogenous-tissue-repairing factors activated by SHED-CM administration could function together to markedly improve liver injury and survival rates (Hirata et al. 2016, Matsushita et al. 2017).

SHED-CM in Treating Diabetes Mellitus

In a streptozotocin-induced diabetes model in rats, the intravenous administration of human SHED-CM and human BMMSCs-CM resulted in pancreatic β-cell regeneration, with elevated insulin secretion in the SHED-CM group. This antidiabetic effect of SHED-CM was found to be superior as compared to BMMSCs-CM (Izumoto-Akita et al. 2015).

SHED-CM in Treating Immunological Disorders

In a rheumatoid arthritis rat model, SHED-CM or BMMSCs-CM intravenous injection markedly improved arthritis symptoms and decreased joint destruction, particularly in the SHED-CM group. This therapeutic efficacy was attributed to the anti-inflammatory capacity of SHED-CM associated with the induction of M2 macrophage polarization and the inhibition of osteoclastogenesis (Ishikawa et al. 2016). Moreover, SHED-EXs were effective in suppressing inflammation and maintaining anabolism homeostasis in temporomandibular joint chondrocytes' cell culture treated with proinflammatory factors (Luo et al. 2019).

Similarly, paracrine factors such as chemokines, cytokines and growth factors secreted by the SHED-CM may play a key role in regulating human hair growth. Human SHED-CM showed promising results in the treatment of alopecia. In an in vivo study, the dorsal area of mice was shaved and was injected subcutaneously with human SHED-CM or human hair follicle stem cells-CM (HFSCs-CM). SHED-CM resulted in a faster stimulation of hair growth compared to HFSCs-CM through upregulating positive hair growth regulatory factors, stromal-cell-derived factor (SDF)-1, hair growth factor, PDGF-BB, and VEGF-A (Gunawardena et al. 2019).

SHED-CM in Treating Dental-Pulpal Disorders

Cytokines commonly expressed by SHED-CM are involved in various mechanisms, including the regeneration of the dentin-pulp complex and vascularization. The angiogenic effect of SHED-CM was studied in vitro on human umbilical vein endothelial cell (HUVEC) culture and in vivo on rats' dental pulp. An inductive effect was observed in HUVEC cultures, indicating that SHED-CM has a pro-angiogenic influence, suggesting that these cells can enhance the vascularization of regenerated dental tissues. Endodontic treatment was performed on rats' first molar tooth, followed by overinstrumentation to allow the blood clot formation to fill the root canal, then SHED-CM was applied on top of the blood clot. Vascular connective tissues were induced inside the root canal (de Cara et al. 2019). SHED-CM contained higher levels of angiogenic factors compared to bone-marrow-derived MSC-CM (Konala et al. 2020).

SHED-CM in Treating Neural Injuries

SHED-CM contains multiple cytokines and chemokines that have the ability to improve peripheral nerve regeneration and functional recovery (Sugimura-Wakayama et al. 2015). SHED-CM contains MCP-1 and secretes ectodomain of sialic-acid-binding Ig-like lectin-9 (sSiglec-9) crucial for SHED-CM-mediated functional recovery following severe peripheral nerve injury. This unique combination of neurotrophic factors enhances the neurite extension of the peripheral nerve and promotes the formation of a Schwann cell bridge and axonal extension (Kano et al. 2017). In vivo studies also showed promising results for SHED-CM in neural regeneration (Kano et al. 2017; Sugimura-Wakayama et al. 2015). SHED-CM administration in a rat nerve gap model was successfully able to induce axon regeneration and remyelination by enhancing the viability and neuritogenesis of neurons of dorsal root ganglia (Kano et al. 2017; Sugimura-Wakayama et al. 2015). MCP-1/sSiglec-9 is considered a set of tissue-repairing M2 macrophage inducers that enhance nerve regeneration by M2 macrophage polarization. This antagonizes the proinflammatory M1 conditions associated with a neural insult (Kano et al. 2017; Matsubara et al. 2015), thereby suppressing inflammatory mediators IL-1β, TNF-α, IL-6, and iNOS and increasing the expression of anti-inflammatory markers arginine-1 and IL-10 (Kano et al. 2017). In a perinatal hypoxia-ischemia-induced brain injury mice model, intracerebral administration of SHED-CM was able to generate an anti-inflammatory microenvironment, which reduced tissue loss and resulted in significant recovery in neurological function, survival rate, and neuropathological score (Yamagata et al. 2013). In a further investigation, SHED-EXs enhanced the recovery of focal cerebral injury in rats (Inoue et al. 2013), improved rat motor functional recovery, and reduced cortical lesion in a traumatic brain injury rat model (Li et al. 2017). SHED-EXs were not only able to inhibit the activity of the M1 phenotype of microglia but also augmented the activity of the M2 phenotype of microglia, thereby suppressing neuroinflammation by anti-inflammatory cytokines

(Inoue et al. 2013; Li et al. 2017). Moreover, SHED-CM enhanced ischemic brain injury by promoting the migration and differentiation of endogenous neuronal progenitor cells and boosted vasculogenesis (Inoue et al. 2013).

Both SHED-CM and DPSCs-CM significantly promoted the regeneration of transected axons via inhibiting multiple axon growth inhibitor signals directly or by paracrine mechanisms, as compared to fibroblast-CM or BMMSCs-CM. In vitro, SHED-CM revealed higher levels of MCP-1 and sSiglec-9 compared with BMMSCs-CM (Sakai et al. 2012). Correspondingly, in vivo, the neuroprotective effects of SHED-CM were confirmed (Asadi-Golshan et al. 2018; Matsubara et al. 2015; Sakai et al. 2012). SHED-CM improved markedly the nerves' functional recovery as compared with BMMSCs-CM (Matsubara et al. 2015, Sakai et al. 2012). The therapeutic effect of SHED-CM was largely attributed to the immunoregulatory functions that activate anti-inflammatory M2-like macrophages and suppress proinflammatory mediators (Matsubara et al. 2015).

Furthermore, SHED-CM converted the proinflammatory brain/spinal cord environment induced by amyloid plaques into an anti-inflammatory state and induced neuroregenerative effects by altering microglial phenotype, as shown in a mouse model of Alzheimer's disease (AD) (Mita et al. 2015) and a mouse model of multiple sclerosis (MS) (Shimojima et al. 2016). SHED-CM administration showed a cognitive function improvement superior to BMMSC-CM and fibroblast-CM despite the similar suppression of the proinflammatory cytokines and the expression of oxidative-nitrosative stress markers attained by SHED-, BMMSC-, and fibroblast-CM. However, SHED-CM uniquely activated M2-type microglia, leading to the expression of the mRNA encoding BDNF, a neurotrophin that plays a key role in synaptic remodeling associated with memory formation. Similar neuropathological recovery was observed in a previous study (Yamagata et al. 2013).

SHED-CM improved functional behavior in an in vitro model of Parkinson's disease. SHED-CM was able to differentiate into dopaminergic neurons. The therapeutic success was attributed to the induction of neurite outgrowth, neuronal survival, together with the repression of 6-hydroxydopamine-induced cell death (Fujii et al. 2015). Similarly, SHED-CM showed a positive outcome in a Parkinson's disease rat model (Jarmalaviciute et al. 2015; Narbute et al. 2019; Chen et al. 2020).

The systemic administration of SHED-CM in treating superior laryngeal nerve lesion rat model protected the swallowing reflex, reduced pharyngeal residue, and promoted functional recovery via two mechanisms (macrophage polarization and vascularization) (Tsuruta et al. 2018).

Collectively, SHED-CM has neural regenerative potential ascribed to the release of multiple growth factors, including NGF, BDNF, NT-3, ciliary neurotrophic factor, and glial-cell-line-derived neurotrophic factor (GDNF) (Sugimura-Wakayama et al. 2015). SHED-CM can stimulate angiogenesis by VEGF expression (de Cara et al. 2019) as well as inhibit iNOS generation (Mita et al. 2015). Taken together, the previous results validated the potential of SHED-CM/EXs as a candidate for neural treatment and the notion that SHED-CM may act through several mechanisms to promote neural functional recovery.

Secretome/Conditioned Medium Derived from Dental Pulp Stem Cells

DPSCs possess a remarkable differentiation potential into ectodermal, mesodermal, and endodermal cell lineages (Yamada et al. 2019a). DPSCs express neural-stem-cell-like markers, besides MSCs markers, like GFAP and nestin, which contribute to the amplification of their self-renewal abilities as well as multipotency (Geng et al. 2017). Moreover, stemness-related markers, like Nanog, octamer-binding transcription factor (Oct)-3/4, and sex-determining region Y (SRY)-box 2 (SOX-2) were also expressed by DPSCs (Yan et al. 2010). The remarkable immunomodulatory properties of DPSCs could be attributed to their ability to express IL-6, IL-8, and TGF-β, which play a role in T-cell functional inhibition (Bianco et al. 2016; Rajput et al. 2014). DPSCs secrete a variety of neurotrophic factors, such as NGF (Zhang et al. 2017b), GDNF (Chang et al. 2014), and BDNF (Kanafi et al. 2014), as well as different angiogenic factors, like VEGF, FGF, and PDGF, with a stimulating increase in their expression following injury (Tran-Hung et al. 2008). Additionally, DPSCs secrete angiogenin, CSF, angiopoietin-1, IL-8, IGF-binding protein-3, and endothelin-1 (Hilkens et al. 2014; Ratajczak et al. 2016; Bronckaers et al. 2013). Despite the fact that DPSCs and SHED are derived from dental-pulpal tissues and have several similar properties, SHED has a higher proliferation rate but a lower osteogenic potential than DPSCs (Yazid et al. 2018). DPSCs, on the other hand, have a higher proliferative capacity and telomerase activity than PDLSCs (Hakki et al. 2015). The previously mentioned characteristics of DPSCs mark their uniqueness, which is further reflected in their secreted secretome/CM.

The Osteogenic Potential of DPSCs-CM

DPSCs osteogenic differentiation may be affected by the surrounding microenvironment (Ma et al. 2009). DPSCs cultivated with DPSCs-CM have enhanced mineralization potential (Paschalidis et al. 2014). The regenerative potential of DPSCs-CM was evaluated in an osteogenic distraction mice model under different culture conditions (Fujio et al. 2017). DPSCs-CM improved the expression of osteogenic and chondrogenic markers, especially under hypoxic conditions. These findings indicate that the paracrine effects of DPSCs upregulate the angiogenic factors (VEGF-A and angiopoietin-2) (Fujio et al. 2017) as well as increase mineralization potential through the expression of TGF-β1 (Paschalidis et al. 2014), which triggers new bone formation and improves osteoblastic/chondrogenic markers (Osterix, SOX-5, factor VIII) (Fujio et al. 2017).

DPSCs-CM in the Treatment of Liver Disorders

One of the promising regenerative applications of DPSCs-CM is the treatment of liver disorders. DPSCs-CM interestingly demonstrated the existence of a variety of

liver lineage proteins, including hepatocyte nuclear factor, oncostatin M (OSM), growth-arrest-specific protein 6, and hepatocyte growth factor receptor in vitro (Kumar et al. 2017b), thereby promoting liver repair and regeneration.

Potential of DPSCs-CM to Regenerate Dental Tissues

EXs derived from DPSCs revealed an effective stimulatory effect on odontoblastic differentiation, in vitro and in vivo, where it prompted the regeneration of dental-pulp-like tissue in an ectopic tooth transplantation model (Huang et al. 2016). Moreover, DPSCs-CM improved the proliferation as well as the migration of fibroblasts (Nakayama et al. 2017) and myoblasts (Kawamura et al. 2016) in vitro. This improvement was confirmed in vivo, through injecting DPSCs-CM into a root and transplanting subcutaneously in an immunodeficiency mice model (Hayashi et al. 2015). DPSCs-CM possesses powerful trophic factors, such as CXCL14 and MCP1, which promoted migration and angiogenesis. Furthermore, combining G-CSF with CM from mobilized DPSCs enhanced the proliferation and migration effects of DPSCs-CM (Nakayama et al. 2017). The high concentrations of BMP or NT-3 in DPSCs-CM (Joo et al. 2018) may be the cause of improving the odontoblastic differentiation of DPSCs in vitro (Murakami et al. 2015). Despite this outstanding potential, DPSCs-CM failed to induce odontoblastic differentiation in cells of nondental origin, like myoblast (Kawamura et al. 2016).

A wide variety of pulp tissue markers were expressed in the tissues regenerated by DPSCs-CM, such as thyrotropin-releasing hormone-degrading enzyme, syndecan-3, G-CSF, CXCL14, neuropeptide Y, BDNF, IL-1α, IL-6, IL-8, IL-16, MCP1 (Hayashi et al. 2015), BMP2, BMP9, PDGF, TGF-β, dentin sialophosphoprotein (DSPP), and runt-related transcription factor 2 (RUNX2) (Huang et al. 2016), in addition to periodontal tissue markers like periostin and periodontal-ligament-associated protein and enamelysin (Kawamura et al. 2016).

Several studies have been conducted to compare the regenerative capacity of DPSCs-CM with other cellular sources. An ectopic tooth model was used to compare BMMSCs-CM, ASCs-CM, and DPSCs-CM in regenerating dental pulp tissues. Compared to other cell-derived CM, DPSC-CM had the highest volume of regenerated pulp tissues. DPSCs-CM has shown angiogenesis in the in vitro dental pulp disease model of HUVEC (Kawamura et al. 2016; Murakami et al. 2015) and embryonic myoblasts (Hayashi et al. 2015) and had antiapoptotic activity in the mouse embryonic fibroblast cell line (NIH3T3) (Ishizaka et al. 2013).

Compared to BMMSCs-CM and ASCs-CM, DPSCs-CM promoted the formation of new blood vessels (Hayashi et al. 2015). Although DPSCs-CM had no significant influence on the proliferation of endothelial cells, it enhanced their migration in vitro (Bronckaers et al. 2013). Furthermore, DPSCs-CM inhibited apoptosis in HUVECs (Iohara et al. 2008) as well as fibroblast cell lines by modulating caspase-3 activity (Nakayama et al. 2017). Several angiogenic factors have been found in DPSCs-CM, including VEGF, IL-8, IGF-binding protein-3, endostatin (Bronckaers et al. 2013), chemokine CXCL 14 (Hayashi et al. 2015), and MCP-1 (Bronckaers et al. 2013;

Hayashi et al. 2015). The aforementioned research highlights DPSCs-CM as a promising new treatment tool for the regeneration of dental tissue through different mechanisms of action, including the promotion of odontoblastic differentiation, antiapoptotic factors, and angiogenesis. Exploring the therapeutic potential of DPSCs-CM in the regeneration of nondental tissues will bring enormous benefits in this era.

The Role of DPSCs-CM in the Treatment of Neurological Diseases

DPSCs-CM showed great potential in neuron regeneration, where it demonstrated the ability to induce recruitment and neuronal maturation, as well as the neuritogenesis of human neuroblastoma cells in vitro (Gervois et al. 2017), along with neurite outgrowth (Ishizaka et al. 2013). The regenerative power of DPSCs-CM, BMMSCs-CM, and ASCs-CM was compared in an in vitro retinal nerve injury model. DPSCs-CM exhibited neuroprotective effects and neurogenesis due to increased levels of different neurotrophic factors (including NGF, BDNF, and VEGF) (Mead et al. 2014). In addition, in an in vitro model of nerve injury, DPSCs-CM promoted the proliferation, differentiation, and migration of Schwann cells and inhibited their apoptosis and increased angiogenesis (Yamamoto et al. 2016).

Additionally, DPSCs-CM displayed a neuroprotective effect in an in vitro model of AD. Its effect was due to the upregulation in the expression of B-cell lymphoma 2 and the downregulation in apoptosis regulator Bax in neuroblastoma cells. In comparison to BMMSCs-CM or ASCs-CM, DPSCs-CM contains a high concentration of fractalkine (antiapoptotic factor), VEGF, and neprilysin, which degrade amyloid peptide (one of the most common misfolded proteins found in AD), besides Fms-like tyrosine kinase receptor-3 (FLT-3), GM-CSF, RANTES, and MCP-1, which makes it a promising candidate for the treatment of AD (Ahmed et al. 2016). DPSCs-CM not only provided a neuroprotective effect but also increased the number and total length of HUVEC tubular structures in an in vitro ischemia model (Song et al. 2017). The systemic administration of DPSCs-CM in a mutant superoxide dismutase mouse model of amyotrophic lateral sclerosis (ALS) showed a promising therapeutic potential (Wang et al. 2019). DPSCs-CM enhanced the neuromuscular junction innervation and survival of motor neurons in treating ALS through various trophic factors and cytokines (Wang et al. 2019). In the same way, when DPSCs-CM was injected into the unilateral hind limb skeletal muscles of diabetic polyneuropathy rats, it demonstrated neuroprotective, anti-inflammatory, and angiogenic effects (Makino et al. 2019). Moreover, in a rat aneurysmal subarachnoid hemorrhage model, the intrathecal administration of DPSCs-CM demonstrated enhancement in cognitive and motor impairments, reduction of neuroinflammation, and microcirculation. Different factors contributed to these significant enhancements, including tissue inhibitors of metalloproteinase (TIMP)-1, TIMP-2, IGF-1, and TGF-β (Chen et al. 2019). Recently, the effect of various manufacturing features, such as the preconditioning of DPSCs with certain

factors, the period of conditioning, and the storage of CM on the functional activity of DPSCs-CM on neurite length, was evaluated (Chouaib et al. 2021). The results revealed that conditioning DPSCs for 48 h is optimal for the functional activity of DPSCs-CM, DPSCs-CM significantly improved neurites' outgrowth of sensory neurons in a concentration-dependent manner, and the frozen storage of DPSCs-CM did not affect the experimental results. Furthermore, the authors demonstrated that conditioning DPSCs with B-27 supplement had a significant impact on the influence of their CM in enhancing neurite outgrowth in primary sensory neurons by altering its growth factors' composition.

Taken together, these data indicate that DPSCs-CM has many neuroprotective and angiogenic factors, such as NGF, BDNF, and VEGF (Mead et al. 2014), RANTES, fractalkine, FLT-3, GM-CSF, MCP-1, and neprilysin (Ahmed et al. 2016), in addition to IGF-1, TGF-β, TIMP-1, and TIMP -2 (Chen et al. 2019). They also provide evidence that DPSCs-CM has a promising ability to induce tissue regeneration in many neurological diseases.

DPSCs-CM in the Treatment of Autoimmune Diseases

Owing to its immunoregulatory properties and anti-inflammatory effects, DPSCs-CM holds a promising potential in the treatment of autoimmune diseases superior to BMMSCs-CM (Yamada et al. 2019b). DPSCs-CM possessed the ability to inhibit allogeneic peripheral blood mononuclear proliferation at different time points (48 and 72 hours after incubation) (Hossein-Khannazer et al. 2020). Recently, DPSCs-CM demonstrated great potential in treating Sjögren's syndrome (a chronic inflammatory autoimmune disease associated with hyposalivation) in a mouse model (Ogata et al. 2021). DPSCs-CM contained extra anti-inflammatory cytokines as compared to BMMSCs-CM. This was reflected histologically by the lesser inflammation in the submandibular salivary gland, in addition to increased salivary flow rate. DPSCs-CM modulated the submandibular salivary gland local inflammatory microenvironment by altering the expression of inflammatory cytokines. The expression of IL-10 as well as TGF-β was increased, while the expression of Il-4, Il-6, and Il-17a was decreased. Furthermore, DPSCs-CM increased the percentage of regulatory T cells while decreasing the percentage of T-helper 17 cells (Ogata et al. 2021). The previously mentioned anti-inflammatory combined with antiproliferative properties of DPSCs-CM makes it a novel cell-free therapy for treating different autoimmune diseases.

Secretome/Conditioned Medium Derived from Gingival Mesenchymal Stem/Progenitor Cells

GMSCs harvested from the gingival connective tissues are MSC subpopulations that possess remarkable regenerative properties (El-Sayed et al. 2015; Jin et al. 2015; Zhang et al. 2012; Fawzy El-Sayed et al. 2012b, 2015). GMSCs are abundant, homogenous, easily obtainable; preserve normal karyotyping; and maintain stable

morphology with passaging. GMSCs have a fast proliferation rate, with remarkable multidirectional differentiation potential and immune regulatory properties (Fawzy El-Sayed and Dorfer 2016). GMSCs could release an array of secretomes with various biological therapeutic actions (Fawzy El-Sayed and Dorfer 2016; Zhang et al. 2012; Fawzy El-Sayed et al. 2018, 2019a; Zhang et al. 2017a; Mekhemar et al. 2018). GMSCs were found to express CD13, CD38, CD44, CD54, CD117, CD144, CD146, CD166, Sca-1, STRO-1, SSEA-4, Oct-3/4, Oct-4A, Nanog, nestin, integrin β1, and vimentin, in addition to MSC surface markers (El-Sayed et al. 2015; Jin et al. 2015; Xu et al. 2013).

GMSCs-CM in Treating Neural Disorders

Various literature pieces suggested that GMSC-derived EXs, EVs, or CM could be an efficient therapeutic approach (Mao et al. 2019; Rao et al. 2019) in managing motor neuron injury (Rajan et al. 2017a), peripheral nerve injury (Mao et al. 2019, Rao et al. 2019), as well as bone (Diomede et al. 2018c) and skin defects (Shi et al. 2017) by increasing the expression of anti-inflammatory cytokine (IL-10), anti-apoptotic cytokine (Bcl2) (Rajan et al. 2017a), and neural-growth-associated markers (BDGF, NT3, neurofilament 200, S100) (Mao et al. 2019) (Rao et al. 2019) (Rajan et al. 2017a) (Zhang et al. 2019), as well as by enhancing the proliferation and regeneration of nerve cells detected by proliferating cell nuclear antigens (PCNAs) (Mao et al. 2019), cell count kit-8 (CCK-8) (Rao et al. 2019), and Shh (Zhang et al. 2019), aside from suppressing proinflammatory cytokines (TNF-α (Rajan et al. 2017a), IL-17, IFN-γ) (Rajan et al. 2016; Giacoppo et al. 2017) and proapoptotic (Bax and cleaved caspase-3) and oxidative stress markers (superoxide dismutase (SOD)-1, iNOS, Cox-2) (Rajan et al. 2017a).

Upon uploading GMSCs-EXs on biodegradable chitin conduits, an enhanced in vitro growth of DRG axon and Schwann cell proliferation, besides a significant increase in the thickness of nerve fibers and myelin sheath and recovery of the muscle and neuromuscular function in rats of sciatic nerve defect model, were revealed (Rao et al. 2019). Similar regenerative results were attained upon using GMSCs-derived EVs in treating crush-injured sciatic nerve in mice. Moreover, GMSCs-EVs robustly blocked the activity of c-JUN/N-terminal kinase (c-JUN/JNK), which abolishes the upregulation of Schwann cell repair genes concomitantly with upregulated the expression of c-JUN, Notch1, GFAP, and SOX-2 genes associated with Schwann cell repair (Mao et al. 2019). The neuroprotective capability of human GMSCs-CM on scratch-injured motor-neuron-like NSC-34 cells was evolved by suppressing apoptotic markers (cleaved caspase-3 and Bax) and oxidative stress markers (SOD-1 and iNOS) while upregulating the expression of anti-inflammatory cytokines IL-10 and neurotrophic factors (BDNF and NT3), in addition to NGF and TGF-β (Rajan et al. 2017a).

GMSCs-EXs proved to regenerate tongue taste buds and papillae after being transplanted in critical-sized tongue defects in rats with an increased expression of $CK14^{+}$; $CK8^{+}$; and types I, II, and III taste bud cell markers (NTPDase 2, PLC-β2,

and AADC, respectively), as well as nerve fiber markers (UCH-L1/PGP9.5 and $P2X_3$ receptor) and two trophic factors (BDNF and Shh), which are involved in the proliferation and differentiation of basal epithelial progenitor cells into taste bud cells and the reconstruction of submucosal connective tissues (Zhang et al. 2019). The rapid wound healing rate in the gingiva was primarily attributed to the GMSCs and their unique secretory mechanism through the Fas/Fas-associated phosphatase-1/caveolin-1 complex, which triggers SNARE-mediated membrane fusion to secrete a large quantity of IL-1 receptor antagonist (IL-1RA)-expressing EVs, inhibiting the proinflammatory cytokine IL-1β (Kou et al. 2018). This finding represents an auspicious application potential for tongue reconstruction in patients suffering from tongue cancer.

GMSCs-CM in Treating Skin Injuries

Isolated EXs derived from GMSCs loaded on chitosan/silk hydrogel sponge effectively promoted the healing of skin defects in diabetic rats. This was evidenced by the formation of neo-epithelium and collagen, an increase in the microvessels' number in the wound bed, and neuronal ingrowth detected by neurofilament heavy chain (NEFH) two weeks postsurgery (Shi et al. 2017).

GMSCs-CM Osteogenic Potential

The osteogenic regenerative potential of a poly-(lactide) (3D-PLA) scaffold supplemented with human GMSCs and human GMSCs-CM was revealed in rat calvaria bone defects after 6 weeks. Moreover, in vitro next-generation sequencing confirmed the increase in the genes involved in ossification (ASF1A, GDF5, HDAC7, ID3, INTU, PDLIM7, PEX7, RHOA, RPL38, SFRP1, SIX2, SMAD1, SNAI1, SOX-9, and TMEM64) in the 3D-PLA loaded with GMSCs-CM (Diomede et al. 2018c). This was attributed to the cytokines and growth factors contained in the CM, which could activate the mobilization and osteogenic differentiation of both endogenous MSCs and GMSCs (Bermudez et al. 2015, 2016; Osugi et al. 2012; Vizoso et al. 2017; Diomede et al. 2018c). In a further study, human GMSCs-EVs that were complexed with polyethyleneimine (PEI) to improve their internalization were loaded on 3D-PLA combined with human GMSCs. The PEI-EVs + 3D-PLA + human GMSCs revealed more calcium deposits with the upregulation of adhesion molecules regulating genes and genes involved in ossification processes, 6 weeks later in vitro. Also, in vivo computed tomography revealed the formation of new bone spicules and blood vessels in rats' calvarial bone defects implanted with 3D-PLA + PEI-EVs + human GMSCs and 3D-PLA + PEI-EVs. It was hypothesized that the osteogenic potential of PEI-EVs-human GMSCs loaded on 3D-PLA was mediated mainly by TGFβR1, SMAD1, BMP2, mitogen-activated protein kinase (MAPK)-1, MAPK14, and RUNX2 through TGF-β signaling (Diomede et al. 2018b).

Recently, it has been hypothesized that the appropriate preconditioning of MSCs with disease-related stimuli can optimize exosomal proteins or miRNA contents, which would efficiently support tissue regeneration and repair. Concomitantly, it has been found that TNF-α preconditioned-GMSC-derived exosomes utilized in treating periodontitis not only enhanced the amount of exosome secreted from GMSCs but also increased the exosomal expression of CD73, thereby inducing anti-inflammatory M2 macrophage polarization. Furthermore, the local injection of GMSC-derived exosomes in a ligature-induced periodontitis mice model markedly reduced periodontal bone resorption and the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts by exhibiting antiosteoclastogenic activity. Such potentials were significantly enhanced by the preconditioning of GMSCs with TNF-α. The previous findings highlighted TNF-α-preconditioned GMSC-derived exosomes as a promising strategy that could alleviate the suffering of patients with periodontitis and other inflammatory osteoimmune diseases (Nakao et al. 2021).

Periodontal Ligaments Stem-Cell-Derived Secretome/ Conditioned Medium

The periodontal ligament (PDL) is considered an enriched source of stem/progenitor cells utilized in periodontal tissue regeneration (McCulloch and Bordin 1991; Isaka et al. 2001; Fawzy El-Sayed and Dorfer 2017; Nagata et al. 2017) due to the enhanced expression of scleraxis (responsible for the formation of the cementum-PDL complex) (Seo et al. 2004). Human PDLSCs are similar to BMMSCs when it comes to immunomodulatory functions, high proliferative rate, and in vitro differentiation ability into adipogenic, osteogenic, chondrogenic, and neurogenic cell lineages (Rajan et al. 2017c; Sedgley and Botero 2012; Achilleos and Trainor 2012). PDLSCs express proteins that are not present in BMMSCs, including CLPP, NQO1, SCOT1, a new isoform of TBB5, and DDAH1 (Eleuterio et al. 2013; Menicanin et al. 2014; Tsumanuma et al. 2011).

The therapeutic effects of human PDLSCs in PDL and alveolar bone homeostasis could be further mediated via secreted paracrine signaling molecules (Rajan et al. 2016). Human PDLSCs were reported to regulate the adipogenic as well as osteogenic differentiation of ABMSCs and inhibit ABMSCs induced osteoclastogenic differentiation of human peripheral blood mononuclear cells (Park et al. 2012). Additionally, PDL cells-CM can regulate the expression of cell proliferation and bone homeostasis genes from MSCs cocultured with BMP-2 (Mizuno et al. 2008).

Permanent and deciduous PDL cells' cytokines profile analysis demonstrated a strong expression of proteins concerned with degradation and immune responses in deciduous PDL-CM, while permanent PDL-CM expressed markedly cytokines related to angiogenesis (EGF and IGF-1) and neurogenesis (NT-3 and NT-4), hence they are a powerful candidate for tissue regeneration (Kim et al. 2016). Moreover, PDL epithelial rests of Malassez demonstrated the expression of significant amounts of chemokines, proteins, and growth factors (IL-1, IL-6, IL-8, and

IL-10; GM-CSF; MCP-1, −2, and −3; amphiregulin; VEGF; GDNF; and IGF-binding protein-2) (Ohshima et al. 2008).

PDLSCs-CM in the Therapy of Neural Disorders

The immunosuppressive effects of human PDLSC secretome in managing MS were proved (Rajan et al. 2016, 2017b) through increased levels of IL-10, TGF-β, and SDF-1α (Rajan et al. 2016). In an autoimmune encephalomyelitis (EAE) model, disease regression and remyelination of the spinal cord were referred to human PDLSC-EX/MV (EMV) fractions. PDLSCs-CM and PDLSCs-EMVs reduced pro-inflammatory cytokines TNF-α, IL-17, IL-6, IL-1β, and IFN-γ and induced anti-inflammatory IL-10 expression, besides attenuated expression of apoptosis-related markers Bax, STAT1, caspase-3, and p53 in the spleen and spinal cord (Rajan et al. 2016). In a more recent study, a downregulated expression of NALP3 inflammasome, cleaved caspase-1, IL-1β, IL-18, Toll-like receptor (TLR)-4, and nuclear factor (NF)-κB was demonstrated in an EAE mouse spinal cord after treatment with human PDLSCs-CM and EMVs. Ultimately, it could be deduced that both human PDLSCs-CM and purified EMVs exerted immunosuppressive effects and may serve as an effective economic approach in treating MS (Rajan et al. 2017b).

Similarly, human PLSCs-CM under hypoxic conditions repressed induced-EAE in a murine model after being injected through the mice's tail vein. The marked expression of antiapoptotic and anti-inflammatory markers (protein Bcl-2 and cytokine IL-37, respectively), as well as the suppression of proapoptotic markers (cleaved caspase-3 and Bax, respectively), was concomitantly associated with the regression of the disease's clinical and histological features. Moreover, a regenerative potential has been observed upon treating the in vitro scratch injury model–exposed neurons NSC-34 via hypoxic-human PDLSCs-CM (Giacoppo et al. 2017). The aforementioned studies propose PDLSCs-CM as a new pharmacologic tool for managing MS through a remarked expression of anti-inflammatory cytokines (IL-10, TGF-β) (Rajan et al. 2016, Giacoppo et al. 2017) and antiapoptotic cytokine (Bcl2) (Rajan et al. 2017a; Giacoppo et al. 2017) and the subsequent suppression of proinflammatory mediators (IL-4, IL-17, IFN-γ, TNF-α, IL-6, IL-1β) (Rajan et al. 2016, Giacoppo et al. 2017), proapoptotic markers (Bax and cleaved caspase-3 (Rajan et al. 2016, 2017a; Giacoppo et al. 2017) and p53 and STAT1 (Rajan et al. 2016)), cleaved caspase-1 (Rajan et al. 2017b), and oxidative stress markers (SOD-1, iNOS, COX-2) (Giacoppo et al. 2017; Rajan et al. 2017a). A reduction in NALP3, IL-1β, IL-18, TLR-4, and NF-κB expression was reported to mediate the nerve regenerative effect of PDLSCs (Rajan et al. 2017b). Moreover, PDLSCs-CM increased the expression of markers associated with neural growth, such as BDNF, IL-37, and NT-3, besides autophagy markers (Beclin-1, LC3) (Giacoppo et al. 2017).

PDLSCs-CM Osteogenic Potential

The in vitro and in vivo results following the culturing of 3D collagen membrane loaded with human PDLSCs and CM or EVs or EVs treated with PEI (PEI-EVs) demonstrated an initially upregulated expression of osteogenic markers (RUNX-2 and BMP-2/4), besides high VEGF, VEGF receptor-2, and collagen type 1 protein levels (Pizzicannella et al. 2019). Likewise, loading Evolution (Evo) (a commercially available collagen membrane) with human PDLSCs enriched with EVs and PEI-EVs revealed high osteogenic properties and biocompatibility in vitro and in rats' calvarial defects. A quantitative reverse-transcription polymerase chain reaction showed the upregulation of osteogenic genes MMP-8, TGF-β1, TGF-β2, tuftelin-interacting protein (TFIP11), tuftelin 1 (TUFT1), RUNX2, SOX-9, and BMP2/4 in the presence of PEI-EVs (Diomede et al. 2018a). Ultimately, these results demonstrated that human PDLSCs might be an effective strategy in bone regenerative medicine, consequent to their potential to increase osteogenic and angiogenic mediators through the TGFβ-BMP signaling pathway.

PDLSCs-CM in Dental Tissue Regeneration

In treating periodontal tissue defects in a rat model, transplanted PDLSCs-CM enhanced periodontal tissue regeneration via suppressing the inflammatory response induced by TNF-α, IL-6, IL-1β, and COX-2. PDLSCs-CM was enriched by extracellular matrix proteins, angiogenic factors, enzymes, cytokines, and growth factors, as revealed by proteomic analysis (Nagata et al. 2017).

Secretome/Conditioned Medium Derived from Stem/Progenitor Cells of Apical Papilla (SCAP), Dental Follicle Stem/Progenitor Cells (DFSCs), and Tooth Germ Progenitor Cells

DFSCs demonstrated proper osteogenic and cementogenic differentiation capacity mediated through the in vitro and in vivo expression of nestin, Notch-1, bone sialoprotein (BSP), collagen type I, osteocalcin (OCN), and fibroblast growth factor receptor (FGFR)1-IIIC (Kemoun et al. 2007; Morsczeck et al. 2005, 2008). Similarly, SCAP possess odontogenic and adipogenic differentiation ability (Abe et al. 2007; Sonoyama et al. 2006) and express neurogenic markers in vitro (Abe et al. 2007). Being the primary source of odontoblasts at the root region, SCAP can differentiate into dentin-pulp complex (Huang et al. 2008). SCAP and DFSCs revealed comparable hepatogenic differentiation potential and superior neurogenic ability to BMMSCs (Rao et al. 2019; Kumar et al. 2017a).

The secretome collected from human DMSCs (DPSCs, DFSCs, and SCAP) stimulated colony formation in preneuroblast cell line IMR-32 and neurite differentiation with a significant increase in neural gene expression (MFI, MAP-2,

β-tubulin III, nestin, and SOX-1), as well as cytokines and growth factors involved in neural regeneration (CSF, IFNγ, TGFβ, NGF, NT-3, and BDNF), more efficiently than BMMSCs' secretome. On the contrary, IL-17 expression was higher in BMMSCs-CM as compared to DPSCs-CM (Kumar et al. 2017a).

DMSCs-CM could further provide a valuable strategy for liver regeneration. The presence of hepatic lineage protein GAS6 in the secretome of DPSCs, SCAP, and DFSCs and different LDL receptor proteins in the secretome of DPSCs and SCAP reflected their role in regulating the transport and metabolism of lipids, as well as hepatic differentiation. Interestingly, OSM and HGFR, important inducers of hepatic lineage differentiation, were expressed solely in DFSC secretome (Kumar et al. 2017b).

The presence of osteogenic lineage proteins was demonstrated in high amounts in human dental MSCs-CM. DPSCs-CM expressed seven proteins, including BMP7 and DSPP, while human DFSCs-CM expressed six proteins, including proteins regulating; endochondral ossification (MINPP1), bone turnover (WISP2) and mineralization (enamelin). SCAP-CM expressed 14 proteins including four of the five proteins expressed by BMMSCs-CM, among them FBN1, DDR2 and Zinc finger protein (ZNF)-423, that play important roles in osteoblastic maturation, activation of BMPs and differentiation of bone osteocytes respectively (Kumar et al. 2018). The ability of DMSCs-CM to express these osteogenic proteins provides a great opportunity for several applications of DMSCs-CM in the regeneration of many bone disorders. The biological effects of dental stem cells conditioned medium are summarized in Fig. 2.

Conclusions

DMSC-derived secretome possesses a diversity of capabilities for tissue engineering and regenerative medicine. The usage of stem/progenitor cell secretome in regenerative medicine provides an appropriate alternative to stem-cell-based therapies with their numerous limitations. Concerning stem/progenitor cells' restrictions, they have a low survival rate after their transplantation (Modo et al. 2002), in addition to a significant risk of malignant transformation following their in vitro expansion, a mandatory step before their clinical use (Baglio et al. 2012; Rubio et al. 2008). A cell-free secretome/CM therapeutic strategy could restore back the function of damaged tissues via the activation of signalling pathways based on the transfer of bioactive molecules, proteins and mRNAs to the affected tissues (Haider and Aslam 2018). Cell-free secretome/CM therapy offers a new perspective in regenerative medicine with minimal or no risks of host rejection, antigenicity, tumorigenicity, and infection, usually present in stem-cell-based therapies.

Despite the numerous benefits of stem cell secretome applications in tissue regeneration, many obstacles are still present before its translation into clinical trials. It is highly recommended to develop a manufacturing, easy-to-apply protocol, free from any animal-based products, as well as determine its proper

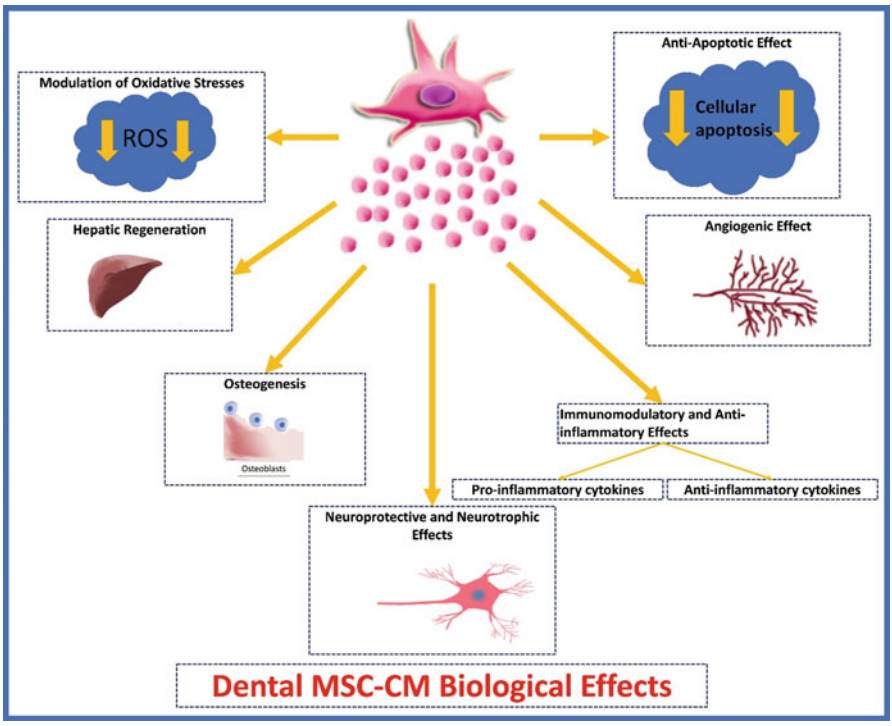

Fig. 2 Illustrative diagram showing dental MSCs-CM biological effect

dosage, exact protein composition, and mechanism of action before applying secretome to human beings. Through emerging technologies and frequent research, the full potential of DMSC secretome in regenerative medicine would be shortly unleashed, and soon it would be ready for its clinical translation into the dental and medical fields.

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Extracellular Vesicles Derived from Mesenchymal Stem Cells

36

Biological Activity and Approaches of Large-Scale Production

M. O. Gomzikova, V. James, and A. A. Rizvanov

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Abstract

The discovery of extracellular vesicles has provided an outstanding breakthrough in stem cells and regenerative medicine. It has been shown that cells can transfer information through the secretion of soluble factors, the formation of direct physical contacts, and the secretion of extracellular vesicles containing a wide range of biologically active factors, including proteins, lipids, and genetic information.

M. O. Gomzikova · A. A. Rizvanov (✉)
Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia
e-mail: marina.gomzikova.gmo@gmail.com

V. James
School of Veterinary Medicine and Science, University of Nottingham, Nottingham, UK
e-mail: victoria.james@nottingham.ac.uk

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All these factors are protected from destruction by the extracellular vesicles' bilayer lipid membrane, allowing the transfer of information from cell to cell over considerable distances. Extracellular vesicles derived from stem cells can reprogram a range of target cells, stimulating their viability and migration. The biological capacity and regenerative potential of stem cell-derived extracellular vesicles are comparable to that of the stem cells in vivo. Due to biosafety concerns over the use of cell-based therapies, the potential of a cell-free therapy based on extracellular vesicles, with equal efficacy to intact stem cells, is highly desirable for future therapeutic purposes. This chapter will discuss current research into the biological activity and therapeutic application of extracellular vesicles derived from mesenchymal stem cells. With a focus on approaches for the large-scale production and isolation of extracellular vesicles will enable the transition of extracellular vesicle research into clinical application.

Keywords

Cell-free therapy · Cytochalasin B · Extracellular vesicles · Hyperosmotic vesiculation · Immunosuppression · Mechanical extrusion · Mesenchymal stem cells · Mitochondria donation · Regeneration

List of Abbreviations

ADSC	Adipose-derived mesenchymal stem cells
AMI	Acute myocardial infarction
BM-MSC	Bone marrow-derived mesenchymal stem cells
CIMVs	Cytochalasin B-induced microvesicles
CKD	Chronic kidney disease
DC	Dendritic cells
dsDNA	Double-stranded DNA
EVs	Extracellular vesicles
GvHD	Graft-versus-host disease
ICG	Indocyanine green
ISEV	The International Society for Extracellular Vesicles
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
miRNA	MicroRNA
MISEV	Minimal information for studies of extracellular vesicles
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
mtDNA	Mitochondrial DNA
MVB	Multivesicular body
MVs	Microvesicles
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
SC	Stem cells
ssDNA	Single-stranded DNA

TNTs	Tunneling nanotubes
UACR	Urinary albumin creatinine ratio
UC-MSC	Umbilical cord mesenchymal stem cells
WJ-MSC	Wharton's jelly-derived mesenchymal stem cells

Introduction

Mesenchymal stem cells (MSCs) have enormous potential in regenerative medicine (Haider and Ashraf 2005). However, the production of cell-based therapeutics is a time-consuming and high-cost process that requires special equipment and expertise. The discovery of the regenerative activity of culture media conditioned by MSCs revealed the role of the MSC secretome in tissue repair and led to the formulation of the paracrine hypothesis (Haider and Aziz 2017; Gomzikova and Rizvanov 2017). MSCs-derived secretome consists of soluble growth factors, cytokines, and extracellular vesicles that mediate the transfer of complex bioactive molecules and the horizontal transfer of genetic information between cells (Lei and Haider 2017). Given the therapeutic significance of the cell secretome, various strategies have been developed to tailor the composition to desired composition (Haider et al. 2008; Durrani et al. 2010; Elmadbouh et al. 2007).

The release of extracellular vesicles (EVs) was first described more than 50 years ago (Wolf 1967). For a long time, it was believed that EVs were inert "garbage" released by cells as a means of off-loading the unwanted waste products. More recently, EVs are being considered as critical components of many physiological and pathological processes and are being considered as essential means of intercellular communication and bioactive material transfer.

EVs are membrane-bound, spherical vesicles released by almost all types of cells. The main functions of EVs are information transfer to recipient cells or to the extracellular environment to mediate intercellular communication (Colombo et al. 2014). EVs are found in almost all tissues, organs, and body fluids: saliva (Palanisamy et al. 2010), urine (Pisitkun et al. 2004), blood (Benz Jr. and Moses 1974), bronchoalveolar lavage (Levanen et al. 2013), nasal discharge (Levanen et al. 2013), amniotic fluid (Keller et al. 2007), uterine fluid (Griffiths et al. 2008), breast milk (Lasser et al. 2011), bile (Witek et al. 2009), cerebrospinal fluid (Street et al. 2012), synovial fluid (Gyorgy et al. 2012), and can also be obtained from supernatants of cultured cells, including MSCs.

EVs contain proteins, lipids, and genetic information obtained from parental cells (Colombo et al. 2014). It is well-established that EVs derived from stem cells retain the same molecular features and demonstrate similar biological activities as the parental cells, such as the presence of specific surface receptors and bioactive molecules. For example, they demonstrate the same potential to stimulate cell proliferation, viability, and chemotaxis of target cells, and suppression of inflammation and oxidative stress (Seo et al. 2019). Thus, the biological effects of MSCs and their secretomes are comparable; however, the secreted products are devoid of a significant number of risks that are otherwise associated with the application of

whole MSC preparations (Lei and Haider 2017). In addition, the composition and biological properties of the secretome as well as EVs secreted by MSCs can be changed in line with tailored therapeutic aims, with no risk of their oncotransformation. To date, there is reported evidence of the regenerative effects of EVs derived from MSCs in the treatment of ischemic and ischemic/reperfusion damage of myocardial cells (Zhao et al. 2019), diabetic complications (Grange et al. 2019), bone defects (Otsuru et al. 2018), liver damage (Li et al. 2013), lung, nervous tissue, and skin injuries (Khatri et al. 2018; Galieva et al. 2019; Ren et al. 2019).

EVs are natural vehicles within the human body, and as such, constitute a promising vector system for the delivery of bioactive molecules and drug targeting. Due to their extremely small size and lipophilic characteristics, EVs demonstrate better tissue distribution and penetration, and are able to successfully cross the blood-brain barrier (Alvarez-Erviti et al. 2011). The ability of EVs to deliver biologically active molecules to the target cells, the absence of nuclei, lack of proliferation and tumorigenicity, biocompatibility, and safety render them a promising therapeutic tool. However, despite encouraging data emanating from the preclinical experimental studies, and clinical trials, currently there are limitations that need to be overcome for their use in the development of EVs-based biotherapeutics. These include the development and optimization of protocols for large-scale production, purification, and storage of EVs. This chapter focuses on the extensive experience of working with EVs besides the in-depth analysis of the published data from other research groups on the development of new drugs and biopharmaceutics based on the secretome products and EVs of human MSCs in the clinical perspective.

Extracellular Vesicles

EVs are spherical structures ranging in size from 50 to 2000 nm, surrounded by a phospholipid bilayer and containing receptors and biologically active molecules, including lipid mediators (e.g., eicosanoids), proteins (e.g., cytokines, chemokines, growth factors, transcription factors, ferments) and nucleic acids (mRNA, rRNA, siRNA, miRNA, tRNA, Y-RNA, ssDNA, dsDNA, mtDNA). It is believed that nucleic acids (especially mRNA, miRNA, noncoding RNA) determine much of the high biological activity of EVs (Haider and Aramini 2020). The composition of noncoding RNAs and their profile found in MSC-derived EVs often differs in the composition found in the MSCs themselves; this indicates the existence of mechanisms of specific RNA loading as well as sorting into vesicles (Groot and Lee 2020; Haider and Aramini 2020). EVs can also capture and transfer whole organelles such as mitochondria (Islam et al. 2012), ribosomes (Court et al. 2008), and proteasomes (Yu et al. 2014; Gomzikova et al. 2019a). The composition of the EV "cargo" depends on the type of the parent cell and its pathophysiological state, culture conditions in vitro, as well as the signals from their microenvironment (Yamamoto et al. 2019).

Cells release different types of EVs into the extracellular space acting on neighboring cells and distant tissues. The range of EVs reported may be broadly categorized as exosomes, microvesicles, and apoptotic bodies (Thery et al. 2018). EVs classification is predominantly based on the route of biogenesis and size (Thery et al. 2018). In addition to the three categories, the biofluid origin EVs are also used to categorize the vesicle types; for example, oviductosomes, prostasomes, epididymosomes, and uterosomes are terms used to indicate vesicles isolated from the oviduct, seminal, epididymal, and uterine fluids, respectively (Machtinger et al. 2016; Al-Dossary et al. 2013). Despite these differences, the critical characteristic of all EVs is the presence of a lipid double-membrane within which bioactive surface molecules are embedded. The International Society for Extracellular Vesicles (ISEV) has developed the Minimal information for studies of extracellular vesicles (MISEV 2018), a comprehensive guide on isolation and classification of different types of EVs (Thery et al. 2018).

Exosomes are small vesicles of endosomal origin with 40–150 nm diameters, which commonly exhibit the markers CD63, CD9, Alix, and TSG101. Exosomes are formed by the invagination of the endosomal membrane into the multivesicular body (MVB), followed by MVB's fusion with the cytoplasmic membrane and release of exosomes into extracellular space (Gomzikova and Rizvanov 2017; Willms et al. 2016).

Microvesicles (MVs) have a larger range of sizes spanning 100–2000 nm in diameter and are characterized by cell type-specific surface receptors, phosphatidylserine and flotillin-2. MVs are formed by protrusion and budding directly from the plasma membrane and carry the cytoplasmic content of the parent cell (Skotland et al. 2020; Gomzikova and Rizvanov 2017). Thus, MVs derived from MSCs retain their surface mesenchymal markers, such as CD44, CD90, CD105, and CD146 (Bruno et al. 2017; Gomzikova et al. 2020a).

Apoptotic bodies are large vesicles with diameters of 1000–5000 nm; they often exhibit annexin V and carry cargos of DNA and histones formed due to programmed cell death and destruction (Yamamoto et al. 2019). These EV subpopulations overlap in size and density, and the cell origin of the EVs can influence the markers they exhibit, making it challenging to isolate pure populations. Therefore, researchers work with a heterogeneous population of EVs more frequently within arbitrary size ranges dictated by the isolation techniques being applied (Gomzikova and Rizvanov 2017).

Mesenchymal Stem Cell-Derived Extracellular Vesicles

MSCs are one of the most promising donor cells for EVs production. They are widespread within an organism and are often found within the bone marrow, adipose tissue, muscles, bones, and umbilical cord blood. They demonstrate low-level immunogenicity due to the low major histocompatibility complex (MHC) class I expression and a lack MHC class II expression (Ryan et al. 2005). EVs derived from MSCs also have inherent low immunogenicity and share the high therapeutic

potential akin to their parental cells, thus rendering EVs a promising therapeutic option in regenerative medicine.

The main advantage of EVs in general and exosomes in particular over soluble factors is that they carry a spectrum of biologically active molecules (proteins, lipids, and nucleic acids) (Haider and Aramini 2020). They not only protect their payload, they also transfer this payload to the recipient cells (Fig. 1). In addition, the main advantage of EVs over MSCs is their small size, better tissue penetration, absence of embolism risk, immune acceptance, lack of a nucleus, and the inability to proliferate (Fig. 1) (Gomzikova et al. 2019a).

EVs derived from MSCs promote angiogenesis (Zhang et al. 2015), suppress apoptosis and stimulate cell proliferation (Ren et al. 2019), induce cell migration and tissue regeneration mechanisms (Zhang et al. 2020a), and immunomodulatory activity (Gomzikova et al. 2019a). In addition, MSC-derived EVs can also recruit and reprogram cells required for tissue regeneration (Ratajczak et al. 2006) and deliver biological factors and organelles to the target cells (Gomzikova et al. 2021).

Autologous or allogeneic MSCs can be used for the production of their secretome including EVs for subsequent use in cell-free therapy (Haider and Aslam 2018). Using EVs derived from autologous MSCs is safer and ethically acceptable. However, the reparability and regenerative potential of MSCs significantly decreases with age and chronic diseases such as coronary heart disease and diabetes mellitus (Shahid et al. 2016; Haider 2018; Efimenko et al. 2015). Therefore, the application of EVs derived from allogeneic MSCs may be required to have the necessary therapeutic efficiency and be available as an off-the-shelf "ready-to-use" preparation for immediate clinical needs.

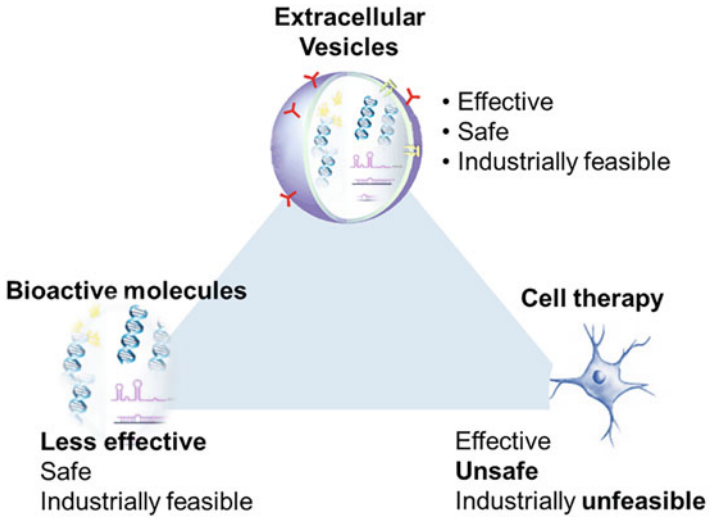

Fig. 1 Advantages and disadvantages of MSCs, MSCs-derived bioactive molecules, and MSCs-derived EVs as therapeutics

Role of Mesenchymal Stem Cell-Derived EVs in Regeneration

An increase in interest in the potential of EVs peaked after the demonstration that EVs derived from MSCs demonstrated biological activity comparable to parental cells. Numerous studies have since shown that MSCs-derived EVs mimic the effects of stem cells in various experimental models of tissue injuries (Table 1).

The beneficial effect of EVs on tissue regeneration is mediated by the stimulation of cell proliferation and migration (Zhang et al. 2020a), inhibition of inflammation (Gomzikova et al. 2019a), and reduction of oxidative stress (Zhou et al. 2013). The use of EVs to stimulate regeneration in cardiovascular diseases has been reported for acute myocardial infarction (AMI), stroke, pulmonary hypertension, and septic cardiomyopathy (Yin et al. 2019). EVs have also been used to demonstrate pro-regenerative activity in treating neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, and nervous tissue injury (traumatic brain injury, spinal cord injuries, stroke) (Galieva et al. 2019). The regenerative potential of EVs was also confirmed in treating liver damage, lung injury, and skin damage (Table 1).

To date, there are three publications on the use of MSC-derived EVs in the clinic. The first evidence of the application of allogeneic EVs in the clinic was obtained in 2014 by Kordelas et al. (2014). The authors administrated MSC-derived EVs to treat steroid-refractory acute graft-versus-host disease (acute GvHD). No side effects were reported and an improvement in GvHD symptoms was seen, including a decrease in IL-1b, TNF-a, and IFN-γ levels (Kordelas et al. 2014). The second was a clinical trial of allogeneic MSC-derived EVs to treat chronic kidney disease (CKD) (Nassar et al. 2016). In 2016, Nassar et al. reported that the administration of MSCs-derived EVs improved the glomerular filtration rate (eGFR), serum creatinine level, blood urea, and urinary albumin creatinine ratio (UACR) in CKD patients (Nassar et al. 2016; Gomzikova et al. 2019a). More recently, the application of MSCs-derived EVs for the treatment of severe pneumonia was observed in COVID-19 patients (Sengupta et al. 2020). The study included 24 patients who received a single intravenous infusion of allogeneic MSC-derived EVs. The use of the EVs was deemed safe as no side effects were evident up to 72 h after infusion (Sengupta et al. 2020). Despite these exciting proof-of-principle studies, further research is needed to evaluate the effectiveness of MSCs-derived EVs, including fully resourced clinical trials with larger patient numbers and adequate control groups.

Given the challenges of producing natural EVs for clinical use, our research group has focused on the production of induced microvesicles derived from MSCs with cytochalasin B. These cytochalasin B-induced MVs (CIMVs) demonstrate identical angiogenic activity in vivo and possess the molecular content and angiogenic activity similar to the parent MSCs (Gomzikova et al. 2019b). This was also demonstrated that CIMVs retain a similar composition of growth factors, cytokines, and chemokines, and express surface receptors of MSCs (CD90+, CD29+, CD44+, CD73+) as the parental MSCs. Importantly, CIMVs can transfer membrane receptors to the surfaces of target cells that might be the mechanism that mediates the mimicry and reprogramming seen as part of MSC-induced regeneration.

Table 1 The regenerative potential of extracellular vesicles derived from mesenchymal stem cells

Source of EVs	Model of disease	Observed effect	Reference
Cardiovascular diseases			
Rat ADSC	Myocardial infarction in rats	Significantly improved cardiac function, suppressed MI-induced myocardial fibrosis and apoptosis, downregulated MI-induced inflammatory factor expression, promoted macrophage M2 t anti-inflammatory polarization, activated S1P/SK1/S1PR1 signaling	Deng et al. (2019)
Adipose-derived regenerative cells (ADRCs)	Myocardial infarction in mice	Prevented cardiac rupture, promoted cardiomyocyte survival by delivering miR-214	Eguchi et al. (2019)
Human UC-MSCs	Myocardial infarction in rats	Repaired the ischemic myocardium by inhibiting cardiomyocyte apoptosis and promoting angiogenesis and ECM remodeling, partly by activating the prosurvival Akt/Sfrp2 pathway	Ni et al. (2019)
Mouse BM-MSCs	Myocardial ischemia/ reperfusion model in mice	Attenuated myocardial injury via shuttling miR-182, shifted polarization of macrophages to M2 phenotype	Zhao et al. (2019)
Rat BM-MSCs	Myocardial ischemia–reperfusion injury in rats	Inhibited cardiomyocyte apoptosis, downregulated PTEN level, activated the PI3K/AKT signaling pathway, protected injured cardiomyocytes via miR-486-5p	Sun et al. (2019)
Liver diseases			
Human UC-MSCs	CCl4-induced liver fibrosis in mice	Decreased collagen expression and Smad2 activity, anti-fibrotic and anti-inflammatory effects	Li et al. (2013)
Human UC-MSCs	Acute liver failure induced by LPS + D-GalN in mice	Decreased levels of NLRP3, caspase-1 anti-inflammatory cytokines	Zhang et al. (2020b)
Lung injury			
Mouse BM-MSCs	LPS-induced acute lung injury in mice	Suppression of signaling pathways of inflammation, reduction of SAA3 expression – Reduction of edema	Yi et al. (2019)

(continued)

Table 1 (continued)

Source of EVs	Model of disease	Observed effect	Reference
Swine BM-MSCs	Influenza virus in pigs	Inhibited the hemagglutination activity of influenza viruses, decreased apoptosis in lung epithelial cells, reduced production of pro-inflammatory cytokines, alleviated lung lesions	Khatri et al. (2018)
Kidney diseases			
Rat MSCs	Renal ischemia reperfusion injury in rats	Attenuated pathological damage, inhibited the inflammatory response, inhibited NF-κB activation, inhibited the expression levels of cleaved caspase-3, caspase-9, and Bax, and upregulated expression level of Bcl-2	Galieva et al. (2019)
Human UC-MSCs	Nephrectomy in rats	Reduced cell apoptosis and enhanced proliferation, renal function was improved and the histological lesion was mitigated, increased capillary vessel density and reduced renal fibrosis	Zou et al. (2016)
Neurological diseases			
Human BM-MSCs	Autoimmune encephalomyelitis in mice	Reduced demyelination, decreased neuroinflammation, upregulated the number of Tregs, reduced levels of pro-inflammatory cytokines, increased levels of anti-inflammatory and neuroprotective proteins	Riazifar et al. (2019)
Human ADMSCs	Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease, a progressive model of multiple sclerosis in mice	Improved motor deficits, reduced brain atrophy, increased cell proliferation in the sub-ventricular zone and decreased neuro-inflammation	Laso-Garcia et al. (2018)
Human BM-MSCs	Traumatic brain injury in rats	Improved cognitive and sensorimotor functional recovery, increased the number of newborn neurons and endothelial cells, reduced neuro-inflammation	Zhang et al. (2017)
Human WJ-MSC	Perinatal brain injury in rats	Reduced neuro-inflammation, decreased expression of inflammation-related genes and secretion of pro-inflammatory cytokines by glial cells	Thomi et al. (2019)

(continued)

Table 1 (continued)

Source of EVs	Model of disease	Observed effect	Reference
MSCs	Spinal cord injury in rats	Improved the functional recovery, reduced neuron loss, delivered miR-21 inhibiting the neurons apoptosis	Kang et al. (2019)
Human ADMSCs	Sciatic nerve transection and implantation in rats	Promoted Schwann cells proliferation, migration, myelination, and secretion of neurotrophic factors in vitro, improved axon regeneration, myelination, restoration of denervation muscle atrophy in vivo	Chen et al. (2019)
Rat ADMSCs	Subcortical stroke in rats	Improved long-term functional outcome, enhanced axonal repair and brain connectivity, reduced cell death, reduced astrogliosis, decreased GFAP expression	Otero-Ortega et al. (2020)
Skin damage			
Human BM-MSCs	Excisional wounds in streptozotocin-induced diabetic rats	Activated the PI3K/AKT signaling pathway, enhanced wound healing, stimulated angiogenesis in vivo	Ding et al. (2019)
Human UC-MSCs	Full-thickness skin defects in mice	Suppressed myofibroblast formation inhibiting the TGF-β2/SMAD2 pathway	Fang et al. (2016)
Human ADMSCs	Full-thickness skin defects in mice	Promoted proliferation, migration, and angiogenesis, upregulated gene expression of proliferative markers and growth factors in vitro, increased reepithelialization, collagen deposition, neovascularization, and wound closure in vivo	Ren et al. (2019)

BM-MSCs bone marrow-derived MSCs, *ADMSCs* adipose-derived MSCs, *UC-MSCs* umbilical cord-MSCs, *WJ-MSC* Wharton's jelly-derived mesenchymal stem cells

Recently, using an experimental model of skin photoaging, it was observed that induced MVs decreased epidermal thickening and improved the organization of the dermal layer (Syromiatnikova et al. 2020). Furthermore, the authors confirmed that CIMVs demonstrate high biological activity and regenerative potential of parental MSCs. Hence, it was proposed that human MSCs-derived CIMVs might be a promising cell-free option for regenerative medicine.

Immunosuppressive Activity of EVs

The immunomodulatory activity of EVs was first described by Raposo et al. The authors showed that EVs derived from B-cells carry MHC class II molecules and participate in the regulation of T-cell response (Raposo et al. 1996). Thus, MSCs possess profound immunosuppressive activity, inhibiting T- and B-cell proliferation, modulating regulatory T cell function maturation and activation, as well as influencing antigen presentation by dendritic cells, decreasing the secretion of pro-inflammatory cytokines and cytotoxicity (Gomzikova et al. 2019a).

It was confirmed that MSC-derived EVs also inhibit the activity and maturation of T cells (van den Akker et al. 2018; Khare et al. 2018) and suppress natural killer (NK) cell activity (Table 2) (Di Trapani et al. 2016). Furthermore, MSC-derived EVs can inhibit B-cell activity and direct monocytic cell polarization towards an immunosuppressive M2 phenotype. They also stimulate the differentiation of T-lymphocytes toward an anti-inflammatory phenotype of regulatory T lymphocytes (Tregs) (Table 2) (Zhang et al. 2014; Morrison et al. 2017). The immunosuppressive activity

Table 2 Immunosuppressive effects of MSCs-derived EVs on immune cells

Immune cells	Effect of MSCs-derived EVs	Reference
T-cells	Inhibition of T-cell proliferation	Di Trapani et al. (2016), Blazquez et al. (2014)
	Induction of differentiation toward regulatory T cells	Wen et al. (2016), Del Fattore et al. (2015)
	Upregulation of the immune-modulating factor IL-10	Del Fattore et al. (2015)
B-cells	Inhibition of B-cell proliferation	Di Trapani et al. (2016)
	Inhibition of maturation	Budoni et al. (2013)
	Decreasing of immunoglobulin secretion	
NK-cells	Inhibition of NK-cell proliferation	Di Trapani et al. (2016)
	Reduced NK-cell number	Koch et al. (2015)
Dendritic cells	Suppression of DC activation	Di Trapani et al. (2016)
	Upregulation of immunomodulatory factors (TGF-β and PGE2)	
	Inhibition of DC proliferation	
Macrophages	Inhibition of chemotaxis	Shen et al. (2016)
	Shift the M1/M2 balance	Song et al. (2017)
	Inhibition of M1 (mir-147 dependent)	Spinosa et al. (2018)
	Downregulation of pro-inflammatory signaling (CCL5, TNF-α, and IL-6)	Willis et al. (2018)
	Upregulation of Arg1 (M2-derived)	
	Stimulation of M2 polarization (by delivery of activated signal transducer and astat3)	Zhao et al. (2018)

of EVs has been also described in depth in other published reviews (Gomzikova et al. 2019a).

In numerous preclinical studies, treatment with EVs has been shown to inhibit immune cell proliferation, reduces inflammation, and improves injury symptoms. The immunosuppressive and regenerative effects of EVs derived from BM-MSCs were demonstrated in an experimental model of cardiac ischemia in rats (Teng et al. 2015). The authors showed inhibition of T-cell proliferation in vitro and attenuated infarct size in vivo after the application of EVs (Teng et al. 2015). The use of MSC-derived EVs was also effective in mediating immunosuppression, reducing neuronal degeneration, suppression of microglia activation, and inducing tissue regeneration in experimental animal models of neuronal tissue injury, such as stroke, traumatic brain injury, and acute spinal cord injury (Hu et al. 2016; Drommelschmidt et al. 2017; Ruppert et al. 2018). The immunomodulatory effects of EVs derived from human MSCs in experimental animal models of autoimmune diseases also demonstrated a range of beneficial effects, including a decrease of inflammatory infiltrates and brain atrophy coupled with an increase in cell proliferation in experimental model of multiple sclerosis (Ruppert et al. 2018). In mouse model of type 1 diabetes, EVs caused inhibition of antigen-presenting cell activation and suppression of Th1, Th17 (Shigemoto-Kuroda et al. 2017). For experimental models of rheumatoid arthritis, inhibition of T-lymphocyte proliferation and attenuation of inflammatory response have been reported (Cosenza et al. 2018). In vivo models of atopic dermatitis showed an improvement of the symptoms, with decreased expression of inflammatory cytokines and reduced eosinophils, mast cell infiltration, and CD86+, CD206+ cells in the skin under the area of atopic dermatitis (Cho et al. 2018). Finally, EVs improved symptoms and decreased mortality in recipient mice undergoing acute graft versus host disease (Wang et al. 2016; Fujii et al. 2018). Furthermore, the authors showed that the use of EVs led to a decrease of CD4+ and CD8+ T-lymphocytes, as well as suppression of T-lymphocyte differentiation into the effector phenotype and an overall reduction in organ damage (Wang et al. 2016; Fujii et al. 2018).

The immunosuppressive activity of the induced microvesicles (CIMVs) derived from MSCs have also been demonstrated (Gomzikova et al. 2020b). The data showed that CIMVs inhibited phytohemagglutinin (PHA)-induced proliferation of peripheral blood mononuclear cells (PBMCs), with a more pronounced effect on T-lymphocytes. Moreover, CIMVs significantly suppressed activation of T-helpers (CD4 + CD25+), B-cells (CD19 + CD25+), and T-cytotoxic lymphocytes (CD8 + CD25+) in vitro (Gomzikova et al. 2020b). Using an experimental model of mice immunization with ovine red blood cells, it was demonstrated that MSCs-derived CIMVs suppressed the humoral immune response and antibody production in vivo (Gomzikova et al. 2020a). Interestingly, no immunosuppression was observed in the animals pretreated with MSCs-derived EVs. In contrast, MSCs themselves and CIMVs-derived from them were similarly effective in suppressing antibody production in vivo (Gomzikova et al. 2020a). Therefore, the authors proposed that CIMVs are potentially a more appropriate substitutes for the MSCs-based cell therapy than endogenously produced EVs, combining advantages of safety and ease of production with retaining the parental MSCs immunomodulatory activity (Gomzikova et al. 2020a).

EV-Mediated Mitochondria Donation by MSCs

The regenerative activity of MSCs is accomplished by a complex influence on the injured cells. This includes the aforementioned stimulation of cell migration and proliferation, besides immunosuppression. More recently it is becoming widely accepted that the regenerative effect of MSCs is also in part mediated by the donation of mitochondria to recipient cells. Mitochondria donation from MSCs leads to the rescue of injured cells, improved oxidative phosphorylation, increased ATP production, and restoration of mitochondrial function (Gomzikova et al. 2021). Moreover, MSCs can also regulate the activity of immune cells through mitochondria transfer (Luz-Crawford et al. 2019; Gomzikova et al. 2021). The most recent studies to evidence mitochondrial transfer from MSCs to recipient cells are summarized in Table 3.

Mitochondria transfer can be via cell-to-cell-based or cell-free mechanisms. The cell-based mechanism includes the transfer of mitochondria through tunneling nanotubes (TNTs) or cell fusion. The cell-free mechanism is via either the naked transfer of the mitochondria or encapsulation of the mitochondria within EVs (Gomzikova et al. 2021) (Fig. 2). However, due to the risks of cell-based therapy associated with the oncogenic transformation of transplanted cells and their differentiation into undesired cell lineages (Gomzikova et al. 2019a), the cell-free strategies of mitochondrial transfer are more attractive for the development of therapeutic

Table 3 Mitochondria donation from MSCs to recipient cells

Donor cells	Recipient cells, injury model	Effect	Reference
Human marrow stromal cells	Mouse neurons, exposure to hydrogen peroxide	Increased neuronal survival, improved metabolism in vitro	Tseng et al. (2021)
MSCs-derived from iPSCs	PC12 cells, CoCl2-induced hypoxia	Reduced apoptosis and restored δψm, ameliorated mitochondrial swelling, the disappearance of cristae, and chromatin margination in vitro	Yang et al. (2020)
MSCs	Corneal endothelial cells (cecs), 661W cells and ARPE-19 cells	Increased aerobic capacity and upregulation of mitochondrial genes in vitro	Jiang et al. (2020)
MSCs and the MSC cell line HS27	REH, SD1, SEM, and TOM1 cell lines, expose to chemotherapy agents in vitro and in vivo on the model of acute lymphoblastic leukemia (ALL)	Prevents ALL cell apoptosis and death from exogenously administered ROS-inducing agents in vitro	Burt et al. (2019)
BM-MSCs	T helper 17 (Th17) cells, rheumatoid arthritis	Oxygen consumption increase by Th17 cells and interconversion into T regulatory cells in vitro	Luz-Crawford et al. (2019)

(continued)

Table 3 (continued)

Donor cells	Recipient cells, injury model	Effect	Reference
BM stromal cell line	Acute lymphoblastic leukemia cells	Metabolic support, changes in genes related to energy metabolism and redox status in vitro	Usmani et al. (2019)
BM-MSCs	Human umbilical cord vein endothelial cells, cytarabine-induced stress	Reduced apoptosis, promoted proliferation and restored the migration ability and capillary formation in vitro	Feng et al. (2019)
MSCs	Neonatal mouse cardiomyocytes, hypoxia/reoxygenation stress	Anti-apoptosis effect in vitro	Zhang et al. (2019)
MSCs	In vivo: intra-arterial injection on the model of ischemic stroke	Improved mitochondrial activity of injured microvasculature, enhanced angiogenesis, reduced infarct volume, and improved functional recovery in vivo	Liu et al. (2019)
Wharton's jelly MSCs (WJMSCs)	Fibroblasts were isolated from a MELAS patient skin punch biopsy	Mutation burden of MELAS fibroblasts was reduced to an undetectable level, with long-term retention. Improves mitochondrial functions and cellular performance, including protein translation of respiratory complexes, ROS overexpression, mitochondrial membrane potential, mitochondrial morphology and bioenergetics, cell proliferation, mitochondrion-dependent viability, and apoptotic resistance in vitro	Lin et al. (2019)
iPSCs-derived MSCs	Injected into the vitreous cavity of one eye	RGC survival was significantly increased with improved retinal function in vivo	Jiang et al. (2019)
BM-MSCs	Neurons, injection into the spinal cord in vivo, oxygen-glucose deprivation injury in vitro, ischemic injury of the spinal cord in vivo	Improved the bioenergetics profile, decreased apoptosis and promoted cell survival in post-OGD motor neurons in vitro, improved locomotor functional recovery in SCI rats in vivo	Li et al. (2019)
MSCs	Neural stem cells in vitro, intranasal administration in vivo, cisplatin damage in vitro and in vivo	Decreases cisplatin-induced NSC death, reversed decrease in mitochondrial membrane potential in vitro. Prevented the loss of DCX+ neural progenitor cells in vivo	Boukelmoune et al. (2018)

(continued)

Table 3 (continued)

Donor cells	Recipient cells, injury model	Effect	Reference
MSCs	Skin fibroblasts from mitochondrial disease patient in vitro, intravenous injection in vivo	Rescues impaired mitochondrial morphology, enhances host metabolic capacity, and induces widespread host gene shifting in vitro and in vivo	Newell et al. (2018)
BM-MSCs	Jurkat cells, treatment with chemotherapeutic drugs	Increased chemoresistance in vitro	Wang et al. (2018)

approaches. Therefore, mitochondrial transfer into the adult cells is in demand to treat of a range of mitochondria-related diseases, such as diabetes, neurodegenerative diseases, aging, and age-related metabolic disorders.

EV-mediated mitochondria donation by MSCs was first discovered by Islam et al. (Islam et al. 2012). The authors used an experimental LPS-induced acute lung injury model to demonstrate the transfer of mitochondria encapsulated in EVs from bone marrow stem cells (BMSCs) to injured lung alveolar epithelial cells (Islam et al. 2012). Later, Falchi et al. observed membrane vesicles sized between 1 and 8 μm containing mitochondria in cultures of human fetal astrocytes (Falchi et al. 2013). Hayakawa et al. also detected extracellular particles containing mitochondria in cultures of rat cortical astrocytes (Hayakawa et al. 2016). Finally, Phinney et al. confirmed the EVs-mediated mitochondria donation mechanism and suggested that it might be a rescue mechanism following oxidative stress and clearance of mitochondria (Phinney et al. 2015) (Fig. 2).

Recently, it was found that myeloid-derived regulatory cells (Hough et al. 2018), renal scattered tubular cells (Zou et al. 2018), HSCs, B cells, T cells (Zhang et al. 2020c), and neural stem cells (Peruzzotti-Jametti et al. 2020) are also able to release EV-encapsulated mitochondria.

Our research group has demonstrated that induced microvesicles (CIMVs) contain mitochondrial DNA (Gomzikova et al. 2020a), as well as functionally active mitochondria (unpublished data). Therapeutic approaches for delivering functional mitochondria in human cells are being developed that might be applied to treat the deficiency of mitochondrial function in disease.

Approaches of Large-Scale Production of Vesicles

Due to manufacturers and the pharmaceutical industry becoming aware of the potential of EVs, methods for the large-scale production of vesicles are required. Typically, EVs are harvested from conditioned medium derived from MSCs culture. Considering that during 1 h, one cell releases about 50–150 vesicles, mass production using this protocol would be pretty expensive as well as time-intensive (Wan et al. 2018). Initially, one of the most common protocols used for the isolation of EVs was serial centrifugation. This procedure required the high-speed centrifugation of the conditioned medium/liquids

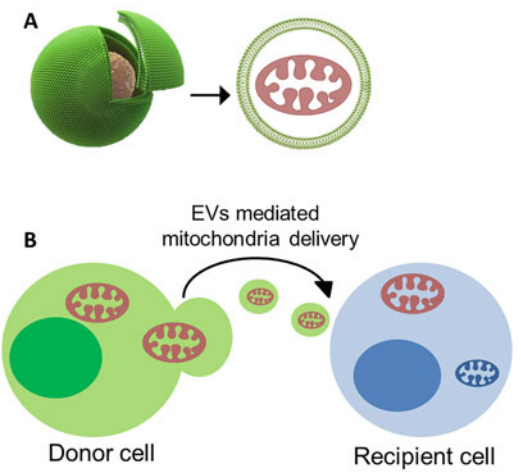

Fig. 2 The scheme of EVs encapsulated mitochondria (*A*) and EVs-mediated mitochondria donation from donor cell to recipient cell (*B*)

containing EVs (up to 100,000–120,000 × g) to precipitate the vesicles in a range of sizes down to 1000 nm or less. The yield of EVs from this method was relatively low and the method itself was labor-intensive and time-consuming. In addition, coprecipitation of protein aggregates, apoptotic bodies, and nucleosomal fragments can reduce the purity of the samples obtained (Momen-Heravi et al. 2013). Fractionation methods such as density gradient centrifugation, ultrafiltration, affinity and gel chromatography, flow field-flow fractionation, and specialized commercial kits have been developed to increase the purity of EVs. However, these methods are often equally time-consuming, expensive, and may require specialist equipment and advanced expertise. Therefore, further development to improve both isolation and large-scale production methods are required before the clinical and commercial benefits of vesicles can be fully developed.

Cytochalasin B-Induced Membrane Vesicles

As previously detailed, treatment of cells with cytochalasin B can induce higher rates of vesicle production (Pick et al. 2005). Cytochalasin B acts to inhibit the polymerization of the actin cytoskeleton (Gomzikova and Rizvanov 2017), causing the increased release of vesicles from the cytoplasmic membrane. This mechanism shares similarities with the natural mechanism of microvesicles production from the cytoplasmic membrane. It is known that for the formation of natural microvesicles, molecular changes within cells are required. The critical event in these changes is the local destruction of the actin cytoskeleton by Ca^{2+}-dependent enzyme calpain (Piccin et al. 2007). Obtained induced microvesicles (CIMVs) surrounded by the cytoplasmic membrane contain the functionally active surface receptors of parental cells (Pick et al. 2005) and have a diameter of 100–1000 nm, comparable

with naturally occurring EVs (Gomzikova et al. 2017). It was found that 17 ± 6% of the cell membrane surface is transformed to induced microvesicles for 1.5 h after treatment with cytochalasin B (Pick et al. 2005; Gomzikova et al. 2020a). These induced microvesicles have been successfully derived from a variety of cell types, including HEK293 (Pick et al. 2005; Mao et al. 2011; Lim et al. 2014), 3T3 fibroblast (Mao et al. 2011), HUVECs (Peng et al. 2015), MDCKII-MDR1 (Eyer et al. 2014), SH-SY5Y (Gomzikova et al. 2017), PC3 cells (Gomzikova et al. 2018), and MSCs (Gomzikova et al. 2020a).

Induced microvesicles have already been shown to have a range of beneficial uses; they have already been employed as bioelectronic sensors of non-small cell lung cancer to detect heptanal in patient plasma (Lim et al. 2014). Induced microvesicles are also promising biocompatible vectors of fluorescence dyes, molecular compounds, and other nanoparticles (Mao et al. 2011). Peng et al. demonstrated that induced microvesicles could be used as a vector to deliver antitumor agents. Following the drug delivery via induced microvesicles, they observed inhibition of tumor growth in an experimental mouse model of xeno-grafted tumors while also reporting that the toxicity of the drug was markedly lower when compared to the administration of the free drug (Peng et al. 2015). Induced microvesicles have also been used to encapsulate ICG (indocyanine green) (Sheng et al. 2016; Gomzikova et al. 2018) and methylene blue (Han et al. 2016). Encapsulation of ICG within the induced microvesicles led to a reduction in the clearance of ICG and increased the effectiveness of photothermal antitumor therapy in vivo (Sheng et al. 2016; Gomzikova et al. 2018). Vesicles loaded with methylene blue showed lower cytotoxicity while maintaining the effect of photodynamic anticancer therapy (Han et al. 2016). Oshchepkova et al. demonstrated that CIMV-encapsulated oligonucleotides are protected from the nucleases and transferred into recipient cells (Oshchepkova et al. 2019).

These data demonstrate that CIMVs inherit the biological activity of parental cells and can be used as cell-free biotherapeutics. Moreover, CIMVs derived from SH-SY5Y cells contain growth factors of the parental cells and can stimulate capillary tube formation in vitro and angiogenesis in vivo (Gomzikova et al. 2017). Additionally, MSCs-derived CIMVs inherit the angiogenic (Gomzikova et al. 2019b), immunosuppressive (Gomzikova et al. 2020a, b), and regenerative activity (Syromiatnikova et al. 2020) of the parental stem cells. Thus, the effective biological activity of CIMVs and ease of loading with desired cargo components (e.g., drugs), together with the easier procedure of production and isolation of a more homogenous population of vesicles makes them a promising therapeutic tool for clinical use and regenerative medicine.

Vesicle Production by Mechanical Extrusion

Membrane vesicles can also be obtained by extruding cells through polycarbonate filters. Wu H.W. et al. proposed to produce membrane vesicles using mechanical extrusion through filters with pore sizes of 1 μm or 2 μm. The authors applied this

method to obtain membrane vesicles from human retinal pigment epithelium (ARPE-19) cells and demonstrated the resulting population of vesicles had diameters 0.2 ± 0.1 μm and 0.8 ± 0.5 μm (Wu et al. 2012). Xu L.Q. et al. applied the same technique using filters with a pore size 3 μm and produced vesicles from BM-MSCs, containing mitochondria (Xu et al. 2017). Despite the ability to produce vesicles of uniform size, the technique risks the degradation of the nuclei and contamination of the vesicles with nuclei material. These factors need to be thoroughly investigated before transferring this technique for mass production and clinical use.

Vesicle Production Using a Hyperosmotic Solution

It was found that osmotic stress induces cells to release vesicles. Del Piccolo et al. developed a protocol based on sequential treatment of cells with hypo- and hypertonic buffers to induce vesiculation (Del Piccolo et al. 2012). Cells are washed twice with 30% PBS in deionized water – a hypotonic buffer that induces cell swelling. Then the cells are placed in a hypertonic solution containing 200 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.75 mM $CaCl_2$ to stimulate vesicle release (Del Piccolo et al. 2012).

Conclusion and Future Directions

The further study of the mechanisms of EV biogenesis, release, and effect on target cells is an important area of research that creates a theoretical basis for their therapeutic application in regenerative medicine.

EVs derived from MSCs carry complex biological cargos and have a range of effects on target cells. However, the full range of these EVs' properties and effects remain to be elucidated. Safe and effective clinical use of EVs requires overcoming several technical difficulties associated with their production. First, it is necessary to develop universal and easily reproducible protocols for the isolation of vesicles, ensuring their high yield with minimal contamination, and improvement in storage methods. Secondly, despite the relative safety of extracellular vesicles administration compared to stem cells, it is necessary to pay close attention to the cultivation conditions of cells (in particular to the presence of animal components in the EV preparations).

EVs derived from MSCs are a promising therapeutic tool, which have advantages over cell-based therapy in terms of safety, ease of storage, and clinical use. In addition, the ability to modify the composition of EVs will open up broader prospects for their use in clinical practice. However, for this potential to be achieved, additional improvements to optimize their production, isolation, and storage are critical before transitioning to the development of biotherapeutics and routine clinical use proceeds.

Cross-References

► Sources and Therapeutic Strategies of Mesenchymal Stem Cells in Regenerative Medicine

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Skeletal Muscle–Extricated Extracellular Vesicles: Facilitators of Repair and Regeneration

37

Facilitators of Repair and Regeneration

Laura Yedigaryan and Maurilio Sampaolesi

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Abstract

Skeletal muscle may be injured upon physical activity, or due to myofiber frailty caused by degenerative disorders. As a metabolic tissue, skeletal muscle has the innate ability of regeneration. Skeletal muscle regeneration may be endowed to

L. Yedigaryan
Translational Cardiomyology Laboratory, Stem Cell and Developmental Biology, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

M. Sampaolesi (✉)
Translational Cardiomyology Laboratory, Stem Cell and Developmental Biology, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Histology and Medical Embryology Unit, Department of Anatomy, Histology, Forensic Medicine and Orthopaedics, Sapienza University of Rome, Rome, Italy
e-mail: maurilio.sampaolesi@kuleuven.be

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the action of quiescent satellite cells, the resident muscle stem cells, and other interstitial and inflammatory cells that directly and indirectly contribute to adult myogenesis. However, the process of muscle regeneration greatly relies on intercellular communication through signaling factors such as proteins, microRNAs (miRNAs), inflammatory cytokines, and membrane lipids that must be tightly coordinated. It is becoming more evident that the release and transmission of these factors involve extracellular vesicles (EVs) liberated by myofibers and other cells in the milieu of the injured muscle. The cargo of EVs is responsible for altering the state of their target cells by delivering purposeful molecules such as messenger RNAs, miRNAs, lipids, and proteins or by aiming at the alteration of gene expression. These changes activate downstream pathways involved in tissue repair. Due to the heterogeneity of EVs with regard to their cargo, location, size, as well as timing of formation and release, the repair and regeneration of skeletal muscle may subsequently be impacted. This chapter focuses on the impact of EVs as biological cues directing stem cell differentiation and modulating the overall process of skeletal muscle regeneration.

Keywords

Epigenetics · Extracellular vesicles · Induced pluripotent stem cells · miRNAs · Mesenchymal stem cells · Muscular dystrophies · Myofiber repair · Satellite cells · Skeletal muscle · Stem cells

Abbreviations

ADMSC	Adipose-derived mesenchymal stem cell
Ago	Argonaute
Akt	Protein kinase B
ASCT2	Alanine-serine-cysteine transporter 2
ASM	Acid sphingomyelinase
BMD	Becker muscular dystrophy
CCL2	C-C motif chemokine ligand 2
CDK2	Cyclin-dependent kinase 2
circRNA	Circular RNA
CXCL1	C-X-C motif chemokine ligand 1
CXCL1	Fractalkine
DMD	Duchenne muscular dystrophy
ECM	Extracellular matrix
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
FAP	Fibro-adipogenic progenitor
GJA1	Connexin 43
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor-1
IL-4	Interleukin 4
ILV	Intraluminal vesicle

iPSC	Induced pluripotent stem cell
lncRNA	Long noncoding RNA
MAB	Mesoangioblast
MDC/CCL22	Macrophage-derived chemokine
miRNA	microRNA
MSC	Mesenchymal stem cell
MVB	Multivesicular body
myomiRs	Muscle microRNA
NO	Nitric oxide
Nox1	NADPH oxidase 1
NRG1	Neuregulin 1 protein
p120	Catenin delta-1
PDGF-α	Platelet-derived growth factor-α
PIC	Twist2s and PW1+ interstitial cell
piRNA	PIWI-interacting RNA
PSC	Pluripotent stem cell
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
S1P	Sphingosine-1-phosphate
SC	Satellite cell
scaRNA	Small Cajal body-specific RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
tRNA	Transfer RNA
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

Introduction

Skeletal muscle represents one of the largest organ systems of the human body. The overall importance of skeletal muscle is attributed to metabolic homeostasis and movement. Therefore, damage to skeletal muscle, resulting in myofiber injury and possible death, highlights the critical ability of skeletal muscle repair and regeneration. Minor injuries may inflict sarcolemmal disruption, which may be repaired by membrane rectification, thus preventing myofiber death. However, severe injuries caused by resistance training overload, heavy load-bearing, and/or genetic defects may cause serious myofiber injury and death. Such injuries are ameliorated by the complex orchestration of inflammatory events and satellite cell (SC) activation and subsequent fusion, constituting the regenerative cycle of skeletal muscle injury. These measures represent the overall method of the restoration attempt upon disruption of homeostasis. Cellular and molecular events during skeletal muscle repair and regeneration constitute complex intracellular and intercellular transactions,

critical for success in the process. Therefore, we will discuss how extracellular vesicles (EVs) – membrane-bound cargoes released by cells, enable communication within and between cells, devising the strict mechanism of repair and regeneration of the injured muscle (Yedigaryan and Sampaolesi 2021; Yedigaryan et al. 2022).

Skeletal Muscle Repair and Regeneration

General Introduction to Skeletal Muscle Regeneration Events

Skeletal muscle regeneration may be classified as a process structured through three distinct yet overlapping phases. Initially, severe injury induces necrosis and significant inflammation. Following the clearance of cellular debris, new fibers expressing embryonic and neonatal myosin heavy chain are formed. Hypertrophy and hyperplasia represent the next step in regeneration, partially regulated by the transforming growth factor-β (TGF-β)/Smad and insulin-like growth factor-1 (IGF-1)/protein kinase B (Akt) pathways. While TGF-β is said to negatively regulate muscle growth, IGF-1 induces muscle hypertrophy by controlling the balance between new protein synthesis and existent protein degradation (Schiaffino et al. 2013). The final step of regeneration is characterized by the restoration of vasculature and innervation patterns. Satellite cells (SCs), adult muscle stem cells, are set-aside between the basal lamina and the plasmalemma of the muscle fibers (Mauro 1961). These cells remain quiescent during homeostasis (Rumman et al. 2015; Schultz et al. 1978); however, upon injury, they proliferate and differentiate into myoblasts that further on fuse to form myotubes (in vitro)/myofibers (in vivo) in order to mature and eventually restore damaged fibers (Fig. 1) (Moss and Leblond 1970; Reznik 1969; Snow 1977; Nakamura et al. 2001).

The maintenance of skeletal muscle during the lifetime of an individual is orchestrated through the action of SCs. As a result of growth cues or physical trauma, SCs become poised for activation. Through symmetric division, SCs reconstitute their quiescent pool. The outcome of asymmetric division is defined through both the repopulation of the SC pool, and the differentiation of some of these cells

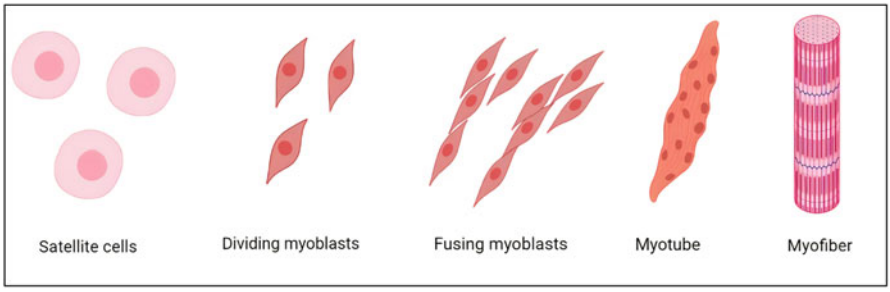

Fig. 1 Progression of satellite cell activation and myotube/myofiber formation. (Created with BioRender.com)

into myoblasts, capable of fusing with each other or damaged fibers to rebuild muscle function and integrity (Baghdadi and Tajbakhsh 2018).

Quiescent SCs are said to express a multitude of genes to uphold their given state (Musarò 2014). The expression of genes such as Hey1 and Hey2 appear to increase once SCs are activated and proliferative (Scharner and Zammit 2011). Pax3 plays an essential role in coaxing quiescent SCs into committing to the myogenic lineage (Relaix et al. 2005; Buckingham 2007). Proliferating SCs express markers such as desmin, Myf5, MyoD, and PCNA (Scharner and Zammit 2011; Creuzet et al. 1998; Yablonka-Reuveni and Rivera 1994). Depending on MyoD expression, SCs may follow two fates. The downregulation of MyoD commits SCs into self-renewal, guaranteeing the regulation of a steady quiescent pool of Pax7-positive SCs. Alternatively, commitment to differentiation is due to the upregulation of MyoD and the consequent downregulation of Pax7, leading to the activation of myogenin expression (Boldrin et al. 2010; Day et al. 2007; Nagata et al. 2006; Relaix and Zammit 2012). Depending on the cellular context, pathways such as Notch and Wnt may either promote or block cell cycle progression. Notch signaling is prevalent during satellite cell proliferation, while Wnt signaling is predominant during differentiation. In some cases, Notch upregulation promotes the transition of activated SCs to proliferative myogenic precursor cells and myoblasts; however, differentiation to myotube formation is stalled. On the other hand, Notch signaling has been demonstrated to be necessary to maintain muscle stem cell quiescence and homeostasis (Bjornson et al. 2012; Mourikis et al. 2012).

Besides muscle injuries, two molecules are said to be responsible for the activation of satellite cells after muscle injury (Relaix and Zammit 2012): hepatocyte growth factor (HGF) and nitric oxide (NO). HGF has been shown to activate quiescent satellite cells into entering the cell cycle both in vitro and in vivo. NO, possibly through the action of metalloproteinases, induces the release of HGF from the extracellular matrix. NO is synthesized through the action of nitric oxide synthase on L-arginine substrates. This component is also said to induce the follistatin expression, a molecule known to antagonize myostatin, thus also possibly contributing to the activation of satellite cells.

The Role of (Other) Residential Cells

Other than SCs, it is clear that different cell types have an impact on the regeneration process. Following muscle injury, pericytes give rise to vessel-associated progenitors called mesoangioblasts (MABs). MABs are progenitor cells derived from the embryonic aorta (De Angelis et al. 1999). These cells are said to contribute to postembryonic mesoderm development and be rooted in the origin of vascular development (Minasi et al. 2002). While MABs have a lower myogenic potential than SCs, their potential for expansion, migration, and regeneration should not be undermined. Another subgroup of pericytes, located peripheral to the endothelium of microvessels, are constituents of the SC niche (Armulik et al. 2011). These cells modulate the behavior of SCs through the excretion of molecules such as IGF-1

(Kostallari et al. 2015). Fibro-adipogenic progenitors (FAPs) that reside in muscle fibers interstitially express markers such as Sca1, CD34, and platelet-derived growth factor-α (PDGFR-α). These cells can differentiate into fibroblasts and/or adipocytes (Joe et al. 2010; Uezumi et al. 2010). In physiological settings, following acute injury, some FAPs are eliminated through apoptosis due to the cues generated by pro-inflammatory cytokines such as interleukin 4 (IL-4) (Joe et al. 2010). However, results have shown that coculture experiments demonstrate the importance of FAPs as sources of pro-differentiation factors. These cues drive the proliferation and differentiation of myoblasts, thus aiding in the positive regenerative outcome. Considering pathological settings, such as muscular dystrophies, FAPs represent the primary source of fibrosis (Lemos et al. 2015). Finally, mesenchymal stem cells (MSCs) are natural precursors of fat, cartilage, and bone (Bianco and Robey 2000). However, MSCs expressing specific transcription factors were reported to act as myogenic progenitors (Liu et al. 2017) and named Twist2s and $PW1^{+}$ interstitial cells (PICs). However, the origin of these cells, as well as the interrelationship among them, remains uncertain.

The Immune Response

Upon muscle injury, neutrophils/monocytes are initially recruited, and their action relies on phagocytosis, oxidation, and proteolysis of necrotic tissue (Sakuma and Yamaguchi 2012; Quattrocelli et al. 2010). Macrophages play a critical role in regeneration, since these cells promote myoblast proliferation.

Despite the nature of the injury, the muscle regeneration process is reprised of two phases: degeneration (necrosis) and reconstruction (Kawiak et al. 2006). In all cases of muscle trauma, damage of the myofibers and sarcolemma consequently results in the permeability of the myofibers. This permeability leads to an influx of calcium into damaged myofibers, activating muscle proteases such as the calpains that damage muscle-important proteins. Leukocytes invade the damaged site as a first step, followed by neutrophils that lyse muscle cells in a superoxide-dependent manner.

Macrophages and Lymphocytes

Initially, neutrophils are recruited, then macrophages (Sakuma and Yamaguchi 2012). Specifically, two different subpopulations of macrophages subsequently invade the injured muscle tissue. The first population (type I [M1]) consists of "inflammatory" macrophages, secreting pro-inflammatory cytokines such as IL-1β. These macrophages are also responsible for the phagocytosis of necrotic tissue. While the "inflammatory" macrophages reach their peak concentration 24 hours after injury, the second population (type II [M2]) of macrophages, the "anti-inflammatory" macrophages, reach their peak at 2–4 days after injury (Mantovani et al. 2007). These macrophages secrete cytokines such as IL-10, known to contribute to the termination of inflammation. This second population of macrophages also has a distinct role in releasing factors that contribute to the proliferation, growth, and differentiation of myogenic precursors. Therefore, it could be elucidated that type I

macrophages are correlated with muscle necrosis, while type II macrophages are associated with regenerative fibers (Pierre and Tidball 1994).

Proper muscle regeneration is permitted through the direct contact of SCs and immune cells (Klimczak et al. 2018). As a result of the secretion of chemotactic factors such as vascular endothelial growth factor (VEGF), macrophage-derived chemokine (MDC/CCL22), and fractalkine (CX3CL1) by SCs, the inflammatory response can be initiated. Eosinophils also play an important role in the innate immune response associated with muscle regeneration (Hoffman et al. 1988; Maeda et al. 2017).

Due to the lack of ability of muscle fibers to conduct a T cell response under normal circumstances, lymphocytes are not involved in skeletal muscle regeneration (Karpati et al. 1988; Maffioletti et al. 2014). However, this is not the case when it comes to inflammatory muscle diseases. In this particular case, muscle cells act as antigen-presenting cells and attract T lymphocytes to the site of injury. These cells are able to express human leukocyte class I and class II antigens (Wiendl et al. 2003).

The Case for Muscular Dystrophy

Muscular dystrophies are a group of genetically heterogeneous neuromuscular disorders associated with progressive muscle weakness and deterioration (Amato and Griggs 2011). The most well-known form of muscular dystrophy is Duchenne muscular dystrophy (DMD). DMD is characterized by the absence of the protein dystrophin. Dystrophin is crucial in upholding the integrity of the sarcolemma, therefore in the absence of this protein, the sarcolemma is rendered unstable and frail (Forcina et al. 2020). Upon contraction, extensive damage of myofibers is apparent and newly regenerated myotubes are not able to rescue the damaged muscle niche. As a result of continuous degeneration, inflammation and regeneration are persistently stimulated, therefore altering the nonpathological dynamic. Concerning the inflammatory response, IL-6 is said to be involved in mediating the unwanted proliferation of SCs while impairing myoblast differentiation (Pelosi et al. 2015; Pelosi et al. 2014; Kurosaka and Machida 2013). Additionally, the transformed behavior of SCs may be dictated by the absence of dystrophin, since daughter cell fate during asymmetric division in dystrophin-deficient SCs may be altered (Chang et al. 2018).

While FAPs assist SCs during nonpathological skeletal muscle regeneration, the role of FAPs in muscular dystrophies turns to mediating fat deposition and fibrosis, promoting the malformed dystrophic microenvironment (Uezumi et al. 2010). The contributions of chronic inflammation, defective myogenesis, and persistent degeneration all lead to the continuous defective regeneration of dystrophic muscles.

Myofiber Intracellular Repair

It is becoming evident that the release and conduction of signaling molecules such as microRNAs (miRNAs) and other noncoding RNAs, proteins, and lipids involve the release of extracellular vesicles (EVs) by myofibers and other nearby cells in the

environment of the injured muscle (Bittel and Jaiswal 2019). Many aspects of EVs are heterogeneous such as the size, location, composition, and time of release. As a result of this, the impact on the repair and regeneration of injured skeletal muscles varies. Vesicular activity is mainly the consequence of the rapid influx of extracellular calcium upon injury and subsequent plasma membrane damage. Specifically, the release of calcium initiates the exocytosis of vesicles such as late endosomes/multivesicular bodies (MVBs) and lysosomes. The release may occur passively or through endosomal sorting complexes required for transport (ESCRT)-mediated release (Andrews et al. 2014; Jimenez et al. 2014; Scheffer et al. 2014; Demonbreun and Mcnally 2017; Romero et al. 2017). The exocytosis of lysosomes aids the endomembrane in closing the wound and allows the release of lysosomal enzyme acid sphingomyelinase (ASM) (Chakrabarti et al. 2003; Jaiswal et al. 2004; Defour et al. 2014; Sreetama et al. 2015), which eventually assists in ceramide release and subsequent plasma membrane repair by removal of damaged membrane through endocytosis and exocytosis (Babiychuk and Draeger 2000; Tam et al. 2010; Corrotte et al. 2013; Romancino et al. 2013; Draeger and Babiychuk 2013). The endocytic vesicles, containing components of the damaged membrane, fuse together to form late endosomes and MVBs (Murphy et al. 2018). Degradation of the internalized damaged proteins and lipids or the inward budding and the consequent creation of intraluminal vesicles (ILV) are the possible next steps. The MVBs may then exocytose their contents. Upon such a fate, ILVs are released and may then be called "exosomes." Not only is the injured plasma membrane repaired, but EVs are able to generate a tissue-level repair response that goes beyond the minute myofiber repair phase. A study has revealed that exercise-induced injury in mice lead to an increase in circulating vesicles, both during the initial hours after injury as well as 5–7 days postexercise injury (Coenen-Stass et al. 2016). This discloses that these released vesicles are produced by both injured myofibers and regenerative cells. Myofibers produce three types of EVs – apoptotic bodies (50–5000 nm in diameter), exosomes (50–150 nm), and ectosomes (microvesicles) (100–1000 nm) (Fig. 2). The difference between these vesicles lies in size, cellular origin, composition, and mechanism of release. Exosomes are the smallest EVs. Other than lipids, exosomes contain proteins such as myogenic growth factors and contractile proteins (Choi et al. 2016; Demonbreun and Mcnally 2017). miRNAs are ~22-nucleotides long noncoding RNAs and mainly inhibit the translation of mRNAs (Bartel 2004) (Fig. 3). In mammals, cleavage of the target mRNA is very rare due to the lack of extensive miRNA: target base pairing (Xu et al. 2016)**.** One example of such an occurrence is the cleavage of HOXB8 mRNA by miRNA-196. By binding to an Argonaute (Ago) protein and forming the core of the multicomponent RNA-induced silencing complex (RISC), miRNAs are guided to anneal to the 3′ untranslated regions (UTRs) of target mRNAs. In response to muscle damage, miRNAs are detected only in vesicles, thus suggesting selective packaging (Siracusa et al. 2016). Moreover, depending on the context: e.g., muscular dystrophy-related damage or muscle injury, the content of specific muscle miRNAs (myomiRs) differs in the released exosomes (Roberts et al. 2012; Matsuzaka et al. 2016; Fry et al. 2017; D'Souza et al. 2018). Currently, more than 1900 different human miRNA sequences have been reported (as of miRBase 22.1, http://www.mirbase.org).

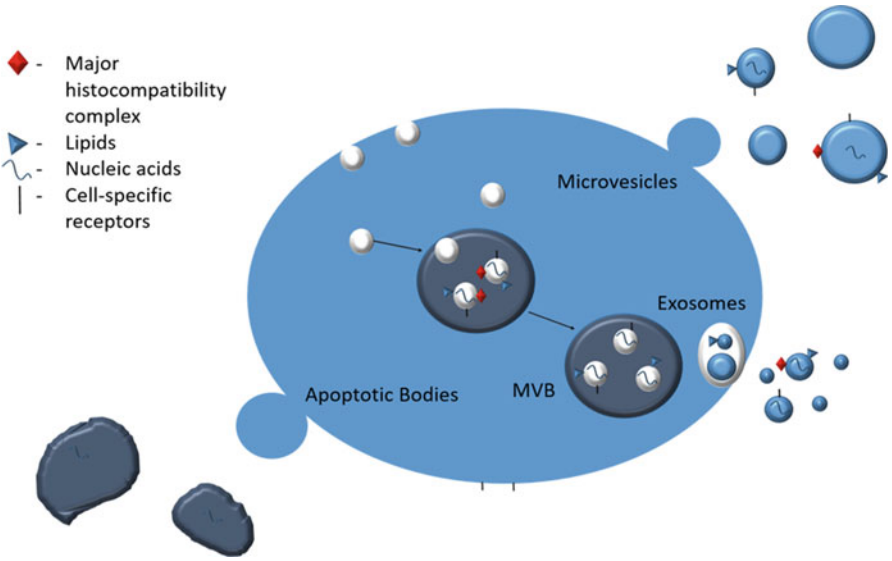

Fig. 2 The synthesis and release of extracellular vesicles (EVs). EVs can be divided into apoptotic bodies, exosomes, and microvesicles

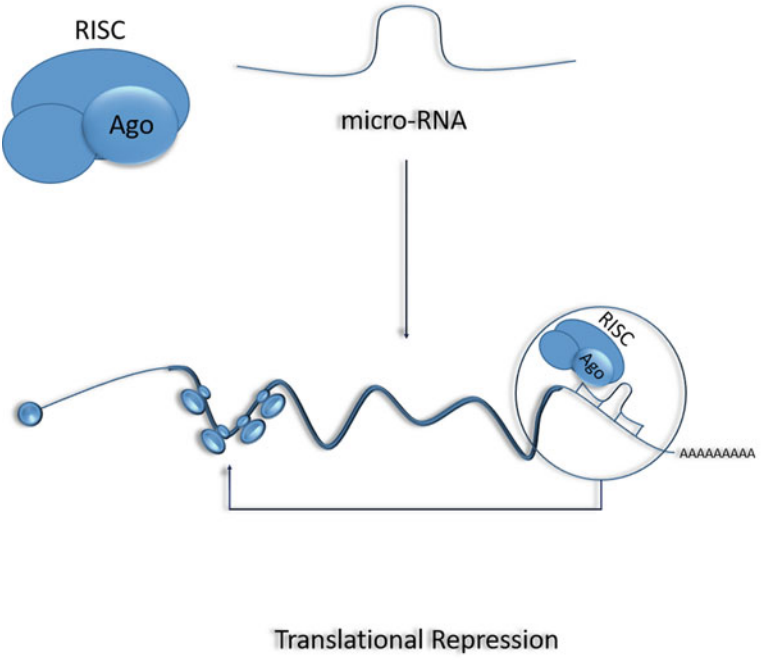

Fig. 3 The mechanism of miRNA and mRNA interaction. The binding of miRNAs, through the guidance of the RISC complex, to target mRNAs induces translational repression

Following muscle damage cycling, exosomal release of miRNA-208a, miRNA-126, and miRNA-16 increases (D'Souza et al. 2018). Similarly, overload-induced injury causes the release of miRNA-206 within SC-derived exosomes (Fry et al. 2017). miRNA-30b and miRNA-181a are said to be involved in muscle regeneration and inflammation as well (Chen et al. 2008; Naguibneva et al. 2006). Lastly, DMD-associated muscle damage leads to the exosomal release of miRNA-1, miRNA-206, and miRNA-133a (Matsuzaka et al. 2016). This is not limited to DMD patients, as increased levels of miRNA-1 were found in limb-girdle muscular dystrophy, Becker muscular dystrophy (BMD), and facioscapulohumeral muscular dystrophy patients (Matsuzaka et al. 2014). Additionally, increased levels of miRNA-133a and miRNA-206 were found in BMD patients. These miRNAs play a vast amount of roles in muscle regeneration and development. The main roles entail the regulation of genes involved in proliferation, myogenesis, and the conversion of muscle fiber type (Cacchiarelli et al. 2010; Cazzella et al. 2012).

In order for skeletal muscle repair to forgo issues, interactions between endothelial cells, inflammatory cells, MSCs, and myogenic stem cells are critical (Wosczyna and Rando 2018). This is facilitated through EVs. EVs are also crucial in the inflammatory response. MSCs release exosomes enriched with miRNA-1, miRNA-133, miRNA-206, miRNA-125b, miRNA-494, and miRNA-601 that promote a myriad of pro-regenerative cellular processes. Other than regeneration, EVs aid in remodeling damaged tissue by facilitating deposition and degradation of the new extracellular matrix (ECM), angiogenesis, fibroblast activation, and tissue cell replenishment. Under pathological settings, such as DMD, secreted vesicles are released with increased levels of miRNAs that promote fibrosis in skeletal muscles and the surrounding ECM (Zanotti et al. 2018).

The ECM is indispensable when it comes to skeletal muscle development. It plays a large role in support as well as signaling (Ishii et al. 2018). Therefore, tissue engineering is a field that is promptly popularized with regard to the potential of inducing skeletal muscle regeneration. Tissue engineering encircles the concept of supporting muscle regeneration by means of three-dimensional implants with preferably the inclusion of scaffolds with bioactive molecules and/or stem cells (Cezar and Mooney 2015; Pascual-Gil et al. 2015; Quattrocelli and Sampaolesi 2015). Hydrogels may be utilized to generate biomimetic skeletal muscle tissues from human-induced pluripotent stem cell (iPSC)-derived cells.

Pluripotent stem cells (PSCs) hold tremendous potential when it comes to cell therapy for degenerative diseases (Judson and Rossi 2020). These cells can essentially give rise to any cell of the adult human body. PSCs are highly proliferative and allow the use of the patients' own cells to avoid immune challenges. In todays' context, it would be of high merit to discuss iPSCs. These cells may be derived by the reprogramming of any human somatic cell, utilizing four transcription factors, namely Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006). When considering the use of these cells in the context of skeletal muscle regeneration, it is important to develop suitable techniques for differentiation into tissue-specific progenitors with properties appropriate for transplantation. A key advantage would

be to derive mesodermal myogenic progenitors from iPSCs as a route for cell therapy for skeletal muscle regeneration. Unfortunately, differentiation of iPSCs into skeletal muscle cells has proven to be challenging. Protocols depicting methods based on transgene mediation as well as soluble factor deployment have been described. Via viral gene delivery, positive results have been obtained in both in vitro and in vivo settings. Ectopic expression of Pax3, Pax7, and MyoD1 produce myogenic cells at high efficiencies (Darabi et al. 2011, 2012; Young et al. 2016; Tedesco et al. 2012; Santoni de Sio et al. 2008). However, much remains to be seen on the effect of intracellular cues as well as the transition through cellular intermediates to mimic natural regeneration. The risk of random viral DNA integration also remains a large concern in the field of cell therapy. Other drawbacks include time, cost, tumorigenicity, and proper quality control.

Extracellular Vesicles

Proteins and Lipids

Considering the origin of exosomes, their lipid content is enriched in lipids from the MVB lipid raft domains (Janas et al. 2015). Lipid rafts contain cholesterols, sphingomyelins, and phosphatidylserine (Choi et al. 2013). Lipid raft–associated proteins suggest the possibility of influence on EV protein sorting. Alternatively, ectosomes contain a more heterogeneous population of lipids (Meldolesi 2018). In addition to lipid content, the protein content difference between exosomes and ectosomes is approximated to be 65% (Le Bihan et al. 2012). EV lipid composition mainly consists of cholesterol, glycosphingolipid, sphingomyelins, phosphatidylserine, and ganglioside GM3 (Choi et al. 2013). Lipids of EVs may induce biological responses such as cell migration and proliferation (Xiang et al. 2018). Target cell interaction may also be influenced by the lipid composition of EVs (Miyanishi et al. 2007; Barreca et al. 2020).

Proteomic analysis of exosomes derived from skeletal muscle revealed the presence of functionally critical proteins such as contractile proteins and myokines (Choi et al. 2016; Demonbreun and Mcnally 2017). On the other hand, ectosomes are enriched in membrane-enraptured proteins. A key mechanism with respect to selective cargo loading during skeletal muscle repair and regeneration may be protein lipid modifications. Protein sorting into exosomes may be affected by intracellular lipid modifications at the MVB membrane, leading to sphingosine-1-phosphate (S1P) receptor activation and Rho-family GTPase stimulation (Kajimoto et al. 2013; Kajimoto et al. 2017). A study done by Ieronimakis et al. revealed that increasing levels of S1P improved muscle regeneration in *mdx* mice (Ieronimakis et al. 2013).

A proteomic characterization of MSCs was performed by Lai et al., and Kim et al. (Lai et al. 2012; Kim et al. 2011). These authors identified specific markers of MSCs, such as CD63, CD109, CD81, as well as surface receptors important for cell differentiation such as EGF-R, and signaling molecules, for instance, RHO and

MAPK1, for cell differentiation and self-renewal. Additionally, proteins implicated in intracellular trafficking, EV biogenesis, fusion, cell adhesion, morphogenesis, and migration were characterized.

RNA Cargo

Unlike the abundance of ribosomal RNAs in the parent cell, EVs are mainly enriched in small RNAs such as miRNAs, long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs) (Crescitelli et al. 2013; Jeppesen et al. 2019). Although miRNAs have been the focus of many studies, a substantial amount of different RNA species are present in EVs (mRNA, ribosomal RNA [rRNA], yRNA, vault RNA, transfer RNA [tRNA], small Cajal body-specific RNA [scaRNA], small nucleolar RNA [snoRNA], small nuclear RNA [snRNA], and PIWI-interacting RNA [piRNA]) that may affect host and/or recipient cells (Kalluri and Lebleu 2016; Li et al. 2014; van Balkom et al. 2015; Zakharova et al. 2007; Vechetti Jr. 2019). Due to the differential RNA sorting mechanism, miRNAs are loaded into exosomes as opposed to ectosomes (Roberts et al. 2012; Matsuzaka et al. 2016; Fry et al. 2017; D'Souza et al. 2018). This is due to EXOmotifs in miRNAs, preferentially sorting them into exosomes (Villarroya-Beltri et al. 2013).

Muscle damage due to muscular dystrophy or eccentric exercise evokes the elevation of tissue-enriched miRNAs (Coenen-Stass et al. 2016). In vivo, this situation is evident during regeneration, while in vitro, myoblast differentiation evokes such a response. After muscle damage, miRNAs are detected only in vesicles, while the level of many circulating miRNAs declines (Siracusa et al. 2016). This reveals the mechanism of selective packaging and release as opposed to unwarranted leakage. Furthermore, depending on the context of muscular damage, miRNAs loaded into vesicles differ and promote or inhibit cell proliferation and/or myogenic differentiation (Table 1) (Roberts et al. 2012; Matsuzaka et al. 2016; Fry et al. 2017; D'Souza et al. 2018). Some microRNAs such as miRNA-1 and miRNA-206 restrict the proliferative potential of these cells, therefore facilitating differentiation. miRNA-1 upholds myogenesis by targeting a transcriptional repressor of muscle gene expression, HDAC4 (van Rooij et al. 2008; Chen et al. 2005). miRNA-206 acts on repressing the expression of Pax7. More specifically, miRNA-206 negatively regulates DNA polymerase α translation, and partakes in the down-regulation of connexin 43 (GJA1) as well as the repression of cyclin-dependent kinase 2 (CDK2) (Callis et al. 2008). Treatment of muscle with miRNA-431, miRNA-675, and miRNA-26a has been shown to increase muscle regeneration since miRNA-431 targets the expression of Pax7, therefore increasing the expression of MRFs in SCs (Wu et al. 2015). On the other hand, some miRNAs are crucial for upholding the integrity of the quiescent population. An example of such is being miRNA-489. It has recently become apparent that miRNAs possess sorting sequences that determine whether they will be secreted into EVs or retained by cells, and that different cell types make preferential use of specific sorting sequences (Garcia-Martin et al. 2021).

Table 1 Muscle-specific miRNAs and possible role(s) in cellular processes

miRNAs	Muscle tissue specificity	Possible role(s)
miRNA-208a	Heart	Promotes muscle fiber shift, regulator of myostatin Promotes muscle growth
miRNA-126		Regulates blood vessel formation
miRNA-16		Regulates blood vessel formation
miRNA-30b		Inflammatory response
miRNA-181a		Promotes myogenic differentiation
miRNA-1/ miRNA-206	Heart/Skeletal muscle, skeletal muscle	Activate satellite cells and promote myogenic differentiation
miRNA-1	Heart/Skeletal muscle	Inhibits cell proliferation, promotes myogenic differentiation, regeneration, angiogenesis regulation
miRNA-206	Skeletal muscle	Inhibits cell proliferation, promotes myogenic differentiation, promotes regeneration of skeletal muscle, promotes regeneration of neuromuscular synapses
miRNA-133a	Heart/Skeletal muscle	Promotes cell proliferation, inhibits cell proliferation, promotes myogenic differentiation, promotes fusion, regeneration, muscle fiber shift
miRNA-133b	Skeletal muscle	Promotes myogenic differentiation and fusion, promotes regeneration
miRNA-125b		Inhibits myogenic differentiation
miRNA-494		Inflammatory response
miRNA-601		Inflammatory response
miRNA-720		Promotes cell proliferation

Uptake of Vesicles by Target Cells

Another important aspect of EVs is the uptake of these vesicles by target cells. The uptake may be done by phagocytosis, macropinocytosis, or receptor-mediated endocytosis. Via different surface receptors and their ligands, exosomes and ectosomes exhibit cell-specific signaling (Sahoo and Losordo 2014). Specific peptides on the EV membrane may prompt EV cargoes to be targeted by neurons or skeletal muscles (Alvarez-Erviti et al. 2011). EVs target different tissues upon skeletal muscle injury and repair. For instance, muscle damage induced by exercise directs EVs to the liver (Whitham et al. 2018). Evidence suggests that the cross talk between other organs may also involve skeletal muscle (Hamrick 2012; Rondon-Berrios et al. 2014). Exosomes are mostly enriched in tetraspanins. Upon muscle injury, exosomes laden with tetraspanins from injured muscle cells aid myoblast fusion. Myoblast spreading and fusion is facilitated by CD9 and CD81 tetraspanins (Hemler 2003). Considering other means of uptake, Syncytin-1-loaded exosomes bind to their transporters' alanine-serine-cysteine transporter 2 (ASCT2), found in skeletal muscle,

to induce internalization (Cocucci and Meldolesi 2015; Kowal et al. 2016). The speed of uptake and the maintenance of elevated amounts of exosomes depends on the circumstance of muscle damage. Depending on the method of EV uptake, different intracellular signaling and sorting fates await vesicle cargoes.

(Rejman et al. 2004; Svensson et al. 2013; Mulcahy et al. 2014; Verdera et al. 2017; Schneider et al. 2017).

EVs Released by Different Cells in the Injured Muscle Environment

EVs are important mediators of intercellular communication that allow the coordinated orchestration of repair and regeneration facilitated by different cell types. The cargoes of EVs affect the recipient cells' mRNA composition and translation. Early stages of injured muscle repair may be characterized by acute actin reorganization and membrane transformation through the action of annexin binding, mitochondrial redox signaling, and ESCRT activity (Jaiswal et al. 2004; Jaiswal et al. 2014; Bouter et al. 2011; Scheffer et al. 2014; Boye et al. 2017; Horn et al. 2017). EVs may be directly involved in some stages of repair. As an example, secretion of Annexin-A1 containing vesicles as a result of epithelial cell injury induces signaling via NADPH oxidase 1 (Nox1), which acts on Rac and catenin delta-1 (p120) proteins that aid in the prompt closure of epithelial wounds (Leoni et al. 2012, 2015). Annexins are crucial not only for myofiber repair but also regeneration. Shedding of vesicles by injured myofibers is enabled by ESCRT-mediated ectosome formation.

The Inflammatory Response via EVs and Subsequent Activation of Nearby Cells

EVs shed by myofibers and other cells also initiate intercellular interactions critical for muscle tissue regeneration. Pro-inflammatory cascades are triggered by EVs through transport of antigens loaded onto major histocompatibility class 1 and 2 complexes to T lymphocytes, as the initial event necessary for triggering the inflammatory response following skeletal muscle injury (Taverna et al. 2017). Neutrophil-derived ectosomes stimulate the release of factors from macrophages for eventual pro-inflammatory macrophage induction (Gasser et al. 2003; Tidball 2017). miRNAs delivered via exosomes reduce the expression of Toll-like receptors (TLR) by macrophages and cause cells to take-up other vesicles without activating the immune response, which has been demonstrated in muscular dystrophy (Manček-Keber et al. 2015; Phinney et al. 2015; Hindi and Kumar 2016).

As stated previously, muscle regeneration is characterized through the clearance of cellular debris and subsequent initiation of the regenerative response (Chazaud 2015). C-X-C motif chemokine ligand 1 (CXCL1) and C-C motif chemokine ligand 2 (CCL2) enrich the muscle environment with cytokines that activate pro-inflammatory macrophages (Tidball 2017). The released cytokines cause

myotubes to produce exosomes that are loaded with myostatin, while also decreasing the level of decorin, a myostatin antagonist (Kim et al. 2018). Therefore, at this pro-inflammatory stage, exosomes participate in limiting myogenesis and allowing the clearance of damaged tissue by inflammatory cells. Consequently, macrophages polarize to the pro-regenerative state with a rise in MSCs. MSCs release vesicles that stimulate myogenin and MyoD, which facilitate regeneration in target cells (Phinney et al. 2015). In addition to this, these exosomes also improve capillary density, attenuate fibrosis, and hasten regeneration of the injured muscle (Nakamura et al. 2015). The cargo of these vesicles includes VEGF, IL-6 in addition to miRNA-1, miRNA-133, miRNA-206, miRNA-125b, miRNA-494, and miRNA-601. These miRNAs promote pro-regenerative cellular developments. Additionally, MSCs can package and transfer mitochondria and mitochondrial proteins within ectosomes to incoming macrophages, in order to maximize their activity for regeneration (Phinney et al. 2015; Sansone et al. 2017).

Skeletal muscle regeneration was found to be enhanced by EVs derived from amniotic fluid MSCs (Mellows et al. 2017; Tsiapalis and O'Driscoll 2020). The effect of adipose-derived mesenchymal stem cells (ADMSCs) on muscle injury demonstrated that repair was facilitated through factors dispersed both within EVs and the secretomes' soluble fraction (Mitchell et al. 2019). EVs derived from MSCs have also been tested as a means to prevent torn rotator cuff injuries (Wang et al. 2019). In a rat model, MSC-EVs prevented inflammation, atrophy, infiltration of fat, and vascularization of muscles, therefore increasing myofiber regeneration and the rotator cuff microenvironmental properties. Recently, ADMSC-EVs have been shown to cease muscle damage in a critical hindlimb ischemia mouse model (Figliolini et al. 2020). This was facilitated through neuregulin 1 protein (NRG1)-mediated signals that play a crucial role in angiogenesis, muscle protection, and inflammation prevention. Lastly, urine-derived MSC-EVs have been shown to repair pubococcygeus muscle injury in rat models of stress urinary incontinence (Wu et al. 2019). This was done through EV-mediated activation, proliferation, and differentiation of SCs. Dendritic cell-derived exosomes and MSC-derived exosomes are being utilized in ongoing clinical trials to treat different types of cancers and graft versus host diseases, respectively (Zhu et al. 2017; György et al. 2015; Jeske et al. 2020).

Exosomes derived from human skeletal myoblasts can induce myogenesis during myotube formation (Choi et al. 2016), reduce collagen deposition, and increase the number of regenerating fibers upon muscle injury (Huang et al. 2016; Campanella et al. 2019). Other types of muscle injury evoke the packaging of alternative components into exosomes. Skeletal muscle denervation shifts exosomal miRNA content from miRNA-133a and miRNA-720 to miRNA-206 which stimulates SC differentiation (Gasperi et al. 2017). SC activation and differentiation increases the secretion of growth factors such as HGF and IGF-1 while simultaneously transferring miRNA cargoes such as miRNA-206 and miRNA-1. SCs also release exosomes that help attenuate fibrosis and enhance myofiber regeneration (Braun and Gautel 2011; Forterre et al. 2013; Choi et al. 2016; Murphy et al. 2018). Muscle injury induced by laceration stimulates the secretion of exosomes enriched in myogenic

growth factors that stimulate the differentiation of adipose-derived stem cells to the myogenic lineage, therefore aiding muscle regeneration (Choi et al. 2016). Oxidatively injured myotubes promote the secretion of vesicles by SCs with cargoes repressing myogenin expression, resulting in faster closure in an in vitro assay (Guescini et al. 2017). Vesicles are also involved in the remodeling of damaged tissue. miRNA-208a regulates fiber-type determination while miRNA-126 and miRNA-16 regulate blood vessel formation (D'Souza et al. 2018). Depending on induced cellular stresses, EVs accomplish specific goals that enable regeneration and remodeling. This is not strictly limited to skeletal muscle, in the early stages of cardiac muscle hypertrophy, the damage promotes cardiomyocyte secretion of miRNA-378-containing EVs. This miRNA plays a role in impairing hyperplasia and the production of collagen (Yuan et al. 2018). Hyperplasia may lead to an increase in heart weight where as a consequence, chronic heart failure, coronary insufficiency, and dilation are noted frequently (Linzbach 1976). Increased collagen synthesis may lead to deterioration of cardiac function and augmentation of myocardial fibrosis (Querejeta et al. 2004).

As a means of therapeutic use, EVs may well be superior to synthetic constructs considering the possibility of bioengineering with the desired factor, as well as the strong biostability and biocompatibility with fewer risks of adverse effects as opposed to stem cell therapy (Riazifar et al. 2017). An interesting consideration for the production of EVs in a scalable mode may be the utilization of PSCs since these cells may be expanded robustly in vitro. From PSCs, more muscle-specific cells such as induced MSC-like cells may be derived and utilized for EV production (Jiang et al. 2019; Steens and Klein 2018; Kim and Kim 2019; Duelen and Sampaolesi 2017).

Conclusion

Recently, it has become more evident that skeletal muscle regeneration does not studiously rely on the propagation of resident stem cells in order to restore homeostatic conditions. The role of extracellular signaling, facilitated by EVs, suggests the existence of EV packaging and networking upon muscle injury induction. The cargo of EVs: proteins, lipids, and nucleic acids play a key role in aiding muscle repair and regeneration. It is evident that depending on the context of muscle injury, the cargo of EVs is custom-engineered by interstitial and immune cells in order to fulfill the necessary intercellular communication and modulate target cells. It is oftentimes thought that regeneration is the outcome of factors secreted by cells, acting in a paracrine manner. The mechanism behind the specificity of EV release and targeting remains an enigma. Current explications emphasize the possibility of EVs either acting through signaling gradients or specific ligand and receptor interactions. Therefore, focusing on such aspects of EVs may unravel their potential in targeted improvement of the injured muscle niche. New insights into the mechanism behind intercellular communication upon muscle injury repair and regeneration may allow future therapeutic achievements, possibly by combining paracrine cues with stem cell therapy.

Even though much work remains to be done in order to establish a proper and standardized means of EV production; by inhibiting their deleterious effects and taking advantage of their regenerative properties, EVs present exciting possibilities in treating muscle-specific disorders.

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Regenerative Medicine Applied to the Treatment of Musculoskeletal Pathologies

38

The Cell-Free Therapy Approach

Alberto González-González, Daniel García-Sánchez, Ana Alfonso-Fernández, Khawaja Husnain Haider, José C. Rodríguez-Rey, and Flor M. Pérez-Campo

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A. González-González · D. García-Sánchez · J. C. Rodríguez-Rey · F. M. Pérez-Campo (✉)
Grupo de Ingeniería de Tejidos de Cantabria, Dpto. Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IDIVAL, Santander, Cantabria, Spain
e-mail: alberto.gonzalezgo@alumnos.unican.es; daniel.garciasa@alumnos.unican.es; josecarlos.rodriguez@unican.es; f.perezcampo@unican.es

A. Alfonso-Fernández
Servicio de Cirugía Ortopédica y Traumatología, Hospital Universitario Marqués de Valdecilla, Santander, Cantabria, Spain
e-mail: ana.alfonso@scsalud.es

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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Abstract

Mesenchymal stem cells (MSCs) have been the most frequent stem cell used in clinical trials due to their easy isolation from multiple adult tissues, their ability to home into the injury sites, and their potential to differentiate into various cell types. Despite this, only two drugs, TEMCELL®, for the treatment of acute graft-versus-host disease, and Alofisel® (Darvadstrocel), for the treatment of fistulae in Crohn disease, have been approved so far by regulatory agencies. For various reasons, MSCs' approval as a drug is proving difficult, and from the outside, it might seem that the field is in a period of stagnation. The risk of lung entrapment or the fact that transplanted MSCs might lead to the formation of tumors are causes of serious concern. Besides, the realization that the beneficial effects of MSCs rely mainly on their paracrine action rather than engraftment and differentiation, has paved the way for cell-free therapeutic strategies in regenerative medicine that would lack the unwanted effects linked to the administration of live cells. The use of MSCs secretome has key advantages over the cell-based therapies, such as lower immunogenicity, and easiness of production, handling, and storage. More importantly, MSCs can be modulated to alter their secretome composition to better suit specific therapeutic goals, thus opening a large number of possibilities. Altogether these advantages are making MSCs secretome the focus of many investigations in several clinical contexts, enabling the rapid scientific progress of this field.

Keywords

Cell-free therapy · Mesenchymal stem cells · Paracrine · Regenerative · Secretome · Stem cells

List of Abbreviations

Agn	Angiogenin
A-MSCs	Adipose tissue-derived MSCs
Ang-1	Angiopoietin 1
bFGF	Basic fibroblast growth factor
COX	Cyclooxygenase
DCs	Dendritic cells
BM-MSCs	Bone marrow MSCs
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EVs	Extracellular vesicles
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1α

HO-1	Heme oxygenase 1
ISCT	International Society for Cellular Therapy
IDO	Indoleamine 2,3-dioxygenase
IL10	Interleukin-10
MCP-1	Monocyte chemoattractant protein 1
MVs	Microvesicles
MS	Mass spectroscopy
MSCs	Mesenchymal stem cells
NK	Natural killer cells
OA	Osteoarthritis
PGE	Prostaglandin E
PlGF	Placental growth factor
ROS	Reactive oxygen species
S-MSCs	Synovial fluid-derived MSCs
SATB2	Special AT-rich sequence- binding protein 2
SDF-1	Stroma cell-derived factor-1
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloprotease 1
VEGF	Vascular endothelial growth factor

Introduction

Mesenchymal Stem Cells Based Therapies: A Useful Tool?

Mesenchymal stem cells, also known as mesenchymal stromal cells (MSCs), have received center-stage attention by both industry and academia due to their unique qualities. Thanks to their regenerative potential and other remarkable properties MSCs are considered excellent candidates for cellular therapy. The escalating research reflects this on their therapeutic applications during the last decade. Since their discovery, more than a thousand clinical trials, many of them interventional, have been registered and reported, while others are in the pipeline. However, the conclusion of these studies has been highly variable, even reporting contradictory results (Couto et al. 2019). Whereas some MSCs-based studies have shown apparent significant beneficial effects, the others did not find an effect at all. This extreme divergence has been generally attributed to the small size of cases analyzed in some trials or the lack of appropriate control groups in the MSCs-based treatments. However, the primary factor responsible for the lack of consistency in these data is considered to be the heterogeneity of the MSCs populations and the quality of cell preparation used for cell therapy. The International Society for Cellular Therapy (ISCT) has established the minimal criteria to define MSCs. According to these criteria, MSCs are fibroblasts-like multipotent adult stem cells that should have the following three properties (Dominici et al. 2006):

1. Adherence to the plastic surface under standard culture conditions.
2. Expression of an array of specific surface markers, that is, CD105, CD73, and CD90, while lacking the expression of hematopoietic specific markers, that is, CD45, CD34, CD79a/CD19, and CD14/CD11b.
3. Multilineage differentiation potential into adipocytes, osteoblasts, and chondrocytes in vitro.

Although this is a useful and valuable definition that initially provided a common ground for describing MSCs, it did not wholly reflect the complexity of MSCs populations that could be gathered from the increasing knowledge about these cells. The isolation of MSCs using the ISCT-devised standard has helped to standardize isolation and purification protocols; however, it still produces heterogeneous populations of cells with diverging potencies, and the standardization of methods remains highly challenging. Efforts are now directed to develop and optimize standard protocols to produce homogeneous MSCs populations to achieve consistent outcomes and optimize the therapeutic use of these cells. One of the options currently tested to achieve this goal is the use of MSCs derived from induced pluripotent stem cells (iPSCs-MSCs). Besides the uniformity of the iPSCs-MSCs cell population, these cells engraft into the recipient tissue and survive at higher rates than the adult tissue-derived MSCs, which render them more appealing and efficient for allogenic transplantation (Gao et al. 2017). It is pertinent to mention that as the investigations involving iPSCs-MSCs have just started, there are still many questions to resolve about their biological characterization, safety, and feasibility-relevant challenges before they could be considered a safe and efficient alternative to adult tissue-derived MSCs.

The heterogeneity of the MSCs' population is not the only drawback hindering the development of MSCs-based therapies. Efficient MSCs-based therapies require MSCs to home, survive, engraft, and integrate with the host tissue at the damaged site. The quality of the cell preparation is an important determinant of cell-based therapy (Shahid et al. 2016). MSCs' performance on these different steps seems to be greatly dependent upon parameters, such as isolation and purification protocols, culture conditions for in vitro expansion, and donor-relevant factors, such as the age of the donor, disease/health status of the donor, or tissue of origin, etc. (Lretlow et al. 2008; Neri 2019; Haider 2018). Furthermore, given the scarcity of these cells in the donor tissues and thus the limited starting material, most of the therapies necessitate a prolonged ex vivo expansion period to obtain enough cells required for transplantation for improved prognosis. The length of this expansion period significantly influences the subsequent treatment outcome (Bertolo et al. 2016) and seems to directly correlate with the loss of the clonogenicity, MSCs' engraftment potential, and differentiation capacity (Thery et al. 2018b). These effects have been linked to a decrease in chemokine receptors expression during the in vitro expansion period, thus translating into a low chemotactic response (Son et al. 2006).

Other factors directly unrelated to the status of the cells, such as the cell delivery method, route of delivery, etc., may also influence the effectiveness of MSCs-based treatments. The treatment strategies involving local administration give superior

outcomes as compared to those involving systemic administration. The reason for this superiority is that the latter leads to the donor cells getting entrapped within the microvasculature of various non-targeted organs, particularly the lungs (Zheng et al. 2016). Even in reaching the target tissue, MSCs will often encounter a highly unfavorable inflammatory and cytotoxic microenvironment characterized by poor perfusion, low oxygen and nutrients, and altered pH, which poses a significant challenge for donor cell survival. This hostile microenvironment often leads to apoptosis of the transplanted MSCs. Some studies have reported as low as 0.1–1 percent survival of the transplanted cells during the first 24 h after delivery, leading to a poor outcome of the procedure. Moreover, the extensive cell death can trigger immune reactions that might aggravate the condition further or even lead to the rejection of the transplanted cells. Given the poor cell survival rate, the transplanted cells could fail to efficiently direct angiomyogenic repair, a crucial step for successful tissue regeneration (Rezaie et al. 2018). Put together, all these obstacles highly reduce the percentage of transplanted MSCs that effectively contribute to the regeneration of the damaged tissue.

It is also important to mention that despite having an excellent safety profile, some published reports have raised a red flag regarding the biosafety of MSCs' use in humans. For example, MSCs have been linked to potential embolisms in small blood vessels (Wu et al. 2017), mainly when the treatment involves systemic administration. The extensive ex vivo expansion required to achieve the cell number necessary for some procedures might contribute to the genomic instability of the transplanted cells. In fact, chromosomal alterations have been observed in clinical-grade MSCs cultures (Nikitina et al. 2018), something worrisome considering that MSCs would maintain their proliferative capacity once in the recipient tissue, increasing the potential risk of tumorogenesis. Besides, although this is rare due to their low immunogenic capacity, it has also been described that the repeated transplantation of MSCs can cause the production of alloantibodies that could limit their clinical applications due to immune rejection possibility (Cho et al. 2008). Despite all the drawbacks mentioned above, and mixed outcomes of MSCs-based treatments, these cells are still contemplated as a highly useful tool and near-ideal cell type from among the cells for cell therapy-based regenerative medicine (Rajab et al. 2019).

The paradigm is now shifting from cell-based therapy to cell-free therapy. Although it was once believed that the main therapeutic benefits of MSCs-based cell therapy primarily relied on their capability to create new tissue via differentiation, growing evidence suggests that the wide range of bioactive molecules secreted by MSCs is responsible for many of the positive effects of MSCs-based therapies. This would explain why, although in many cases less than 1% of the transplanted MSCs are retained long-term within the target tissue, there are still clear therapeutic benefits linked to their transplantation (Yeo et al. 2013). As we will discuss in-depth later in the chapter, MSCs secrete a wide variety of molecules as part of their paracrine activity that has several beneficial effects on the damaged tissue, including their immunomodulatory action, which is considered highly relevant to host tissue cytoprotection and regeneration. Thus, the use of these paracrine secretions, rich in bioactive factors, produced by MSCs could hypothetically replace these cells in

some applications that would also benefit from the lack of biosafety concerns associated with using a cell-free therapy. However, it should be kept in mind that, besides their ability to replace the damaged tissue by differentiating into multiple cell types and their paracrine activity, the therapeutic potential of MSCs could also rely on other mechanisms that would require the physical proximity of those cells.

MSCs exert a cytoprotective effect on the surrounding cells in the host tissue post-engraftment by modulating reactive oxygen species (ROS) production and modifying the nutrient usage mechanistically through intercellular mitochondria trafficking (Paliwal et al. 2018). MSCs also modify the behavior of immune cells by the secretion of specific factors, and cell-to-cell contact (Andreeva et al. 2017; Liu et al. 2020c). Although there is still some controversy in the field, it has been recently shown that MSCs fuse with cancer cells leading, in some cases, to the cessation of their proliferation highlighting the anti-tumorogenic effect of MSCs and opening the opportunity to use MSC-fusion based strategies for tumor biotherapy (Zhang et al. 2020b). These properties of MSCs require direct physical interaction of the transplanted MSCs with the tissue-resident cells. Even though all the aforementioned problems are linked to MSCs-based therapies, these cell-based approaches might still be helpful in treating specific pathologies where these particular activities are proven beneficial.

MSCs Secretome as an Alternative to Cell-Based Therapy

The paracrine activity is a common feature of virtually every cell type. The wide array of bioactive molecules secreted by a particular cell type is designated as the "secretome." This term, initially coined by Tjalsma et al. (2000) who defined it as "both the components of machineries for protein secretion and the native secreted proteins." This definition, as well as the one later provided by Hathout (2007), who defined the secretome as "all the factors secreted by a cell or tissue into the extracellular space under a defined time and conditions," only seem to contemplate the soluble part of this secretome while letting out a key part of it: the extracellular vesicles (EVs).

Several studies have now confirmed that the MSCs' secretome can reduce cell injury and improve tissue repair. Moreover, it has immunomodulatory, anti-apoptotic, and pro-angiogenic properties via activation of endogenous signaling pathways in neighboring cells that would lead to an improved microenvironment and host tissue regeneration. This realization has paved the way for the use of secretome as a cell-free alternative to overcome the limitations of MSCs-based therapies. More importantly, MSCs-derived secretome is being developed as a pharmaceutical product for use in regenerative medicine. MSCs-secretome based products are now being tested as substitutes of MSCs in the applications where those cells have already proven useful (Bogatcheva and Coleman 2019). The use of MSCs-derived secretome has additional advantages over MSCs, such as the ease of handling and storage and off-the-shelf availability. On the same note, the secretome components are highly stable and are not affected by processes such as freezing, concentration by ultracentrifugation, or lyophilization (freeze-drying).

Besides, its production is easily scalable to fit the necessary levels for large-scale production, thus opening the possibility of developing novel MSCs-secretome based pharmacologically active agents.

Although both the soluble and insoluble fractions of the secretome may contribute to tissue regeneration, they might have different effects (Giannasi et al. 2020; Mitchell et al. 2019) and are the base of different preparations currently being tested for therapeutic purposes.

Soluble Factors Secreted by MSCs

Various research groups have attempted to profile and identify the soluble factors secreted by MSCs from different tissue sources using global approaches, including mass spectrometry (MS)-based techniques. However, these analyses have proven to be technically challenging and more often than not, identified false-positive results due to proteins stemming from dead cells or interference from the culture media used. Recently, the use of a highly stringent quantitative MS approach, allowed the identification of 315 proteins actively secreted by human bone marrow MSCs (BM-MSCs) (Baberg et al. 2019). However, it is important to note that despite the secreted paracrine factors from MSCs of different origins being highly similar, MSCs from different tissue sources diverge in the level of production, as recently shown in an experimental animal model (Villatoro et al. 2019). These biological differences should then be considered when choosing the MSCs' source for secretome production.

Proteins secreted by MSCs include cytokines, chemokines, extracellular matrix (ECM) proteases, and growth factors that are involved in a wide range of biological activities in the cells, such as antioxidant and antimicrobial activities, proliferation, cytoprotection, modulation of inflammation, emigrational activity, promotion of angiogenesis, and transdifferentiation. The main activities driven by MSCs soluble factors will be discussed in the following sections. A schematic representation is shown in Fig. 1.

Immunomodulation

Besides other mechanisms, the therapeutic benefits of MSCs are primarily attributed to their immunomodulatory function. MSCs can influence both adaptative and innate immune responses through their secreted factors. These secreted factors exert widespread immunomodulatory effects on virtually all immune cells, including T cells, B cells, dendritic cells (DCs), natural killer cells (NK), neutrophils, monocytes, and macrophages. The main immunosuppressive factors secreted by MSCs are prostaglandin E (PGE), hepatocyte growth factor (HGF), indoleamine-2,3-dioxygenase (IDO), transforming growth factor β (TGF-β), and interleukin-10 (IL10). Interestingly, many of these factors exert their function by interfering with the metabolism of certain amino acids.

MSCs' regulatory effect of macrophage function is mediated mainly through the secretion of PGE2, the synthesis of which is mediated by the constitutively produced

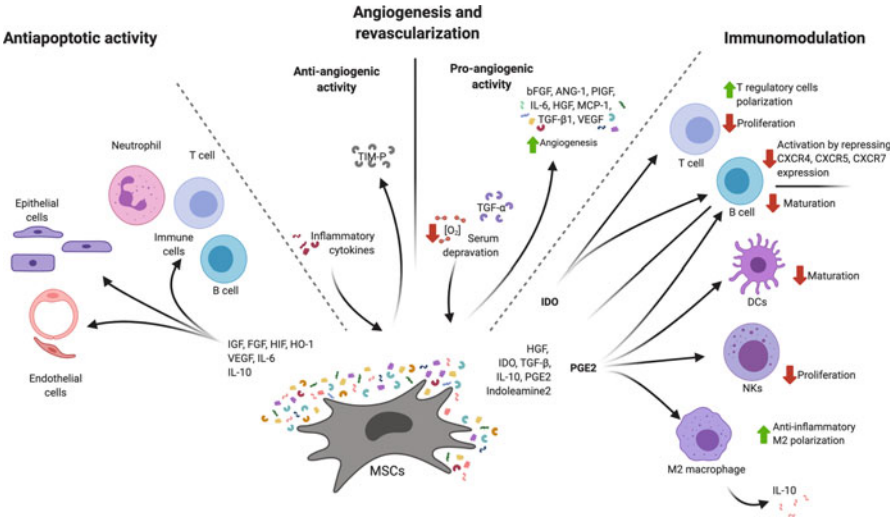

Fig. 1 Schematic representation of MSCs-secreted soluble factors' effect on immunomodulation, angiogénesis, and apoptosis. (Created with **BioRender**.com)

COX1 enzyme. In response to a pro-inflammatory environment, MSCs activate the synthesis of the inducible COX2, directly increasing PGE2 synthesis (Murakami and Kudo 2004).

Macrophages are generated from monocytes through two different pathways, depending on the environment, a classical activation pathway (M1) or an alternative pathway (M2). Both these types of macrophages release a very different set of factors. While M1 macrophages mainly release an array of pro-inflammatory cytokines, M2 macrophages release IL-10, which has a significant anti-inflammatory role. PGE2 induces the differentiation of macrophages toward the M2 anti-inflammatory phenotype (Deng et al. 2016). PGE2 not only regulates macrophage activation but is also able to suppress the proliferation of NK cells and the maturation of B lymphocytes and DCs (Hegyi et al. 2012).

IDO also has important effects on T and B lymphocytes' activation, proliferation, and differentiation. The two isoforms of IDO, namely IDO1 and IDO2, catalyze the degradation of tryptophan needed for T-cell division through the kynurenine pathway. The depletion of tryptophan leads to the arrest of T-cell metabolism and subsequent apoptosis (Böttcher et al. 2016). Furthermore, like the effect on macrophages polarization, T-cell differentiation is also polarized toward T regulatory cells, one of the $CD4^+$ subclasses of T lymphocytes involved in the cellular immune response (Weiss and Dahlke 2019). Regarding B cells, MSCs also affect the humoral activity of these cells by diminishing the expression of chemokine receptors 4, 5, and 7 (CXCR4, CXCR5, and CXCR7), decreasing their overall activation rate.

As mentioned earlier, MSCs can also secrete various pro-inflammatory cytokines. Although this might appear counterintuitive, it is essential to clarify that for proper tissue regeneration, an acute inflammatory response is needed right after the injury,

to allow the migration of MSCs to the injury site and undergo proliferation at a faster rate. However, the subsequent repair phase requires an immunosuppressive state. Therefore, MSCs can sense their microenvironment and respond appropriately in a cue-dependent manner during the healing process (Renner et al. 2009).

Finally, it is necessary to highlight that although the paracrine activity of MSCs is indeed responsible for the immunosuppressive function of MSCs, we also need to clarify that this function is also mediated by cell-to-cell contact and by the mitochondrial transfer to the injured cells via tunneling nanotubes (Cselenyák et al. 2010).

Angiogenesis and Revascularization

MSCs extensively release a variety of pro-angiogenic factors, including basic fibroblast growth factor (bFGF), angiopoietin 1 (Ang-1), placental growth factor (PIGF), Interleukin 6 (IL-6), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β1), and monocyte chemoattractant protein 1 (MCP-1) (Kwon et al. 2014). The presence of all the aforementioned factors and their respective concentrations greatly varies depending upon the MSCs source and their culture conditions (Kehl et al. 2019). The amniotic MSCs secretome shows a more robust angiogenic profile (Kim et al. 2012b). In comparison with the adipose tissue-derived MSCs, human amniotic fluid-derived MSCs were rich in pro-angiogenic factors, including IGF-1, EGF, and IL8, as determined by real-time PCR and enzyme-linked immunosorbent assay (ELISA) (Kim et al. 2012b). The amniotic fluid-derived MSCs were characterized by the surface markers expression of CD44, CD73, CD90, CD105, and CD106, while showing a minimal expression of CD34, CD14, and CD45. Also, while some factors such as bFGF are not always found as part of the soluble factors secreted by different MSCs, other factors, such as IL6, are always present in most of the analyses performed and reported (Hsiao et al. 2012).

Besides the release of pro-angiogenic factors, MSCs also produce anti-angiogenic molecules under a specific set of culture conditions, such as stimulation with inflammatory cytokines. This specific culture environment leads to increased secretion of tissue inhibitor of metalloprotease 1 (TIMP-1), a protein with strong anti-angiogenic activity (Zanotti et al. 2016). A balance between the activity of the metalloproteases and their inhibitors is crucial for the degradation of the vascular basement membrane that prevents endothelial cells from leaving their positions. Together with correct extracellular matrix remodeling, this process is of utmost importance to allow the emigration of endothelial cells toward the surrounding tissue to initiate and participate in the generation of neovasculature.

Hypoxia provides a strong stimulus for the release of pro-angiogenic factors via the activation of hypoxia-inducible factor 1α (HIF-1 α) and its downstream signaling. Therefore, hypoxic treatment of the cells has been extensively studied to alter the secretome profile of the cultured cells (Hsiao et al. 2013; Hao et al. 2019). An augmented secretion of pro-angiogenic factors in the cells may be induced by subjecting the cells to hypoxic culture conditions and by the presence of different cytokines, such as TGF-α, in the culture media. TGF-α increases the secretion of

various angiogenic factors through the activation of PI3K/Akt and MAPK signaling pathways. Conditioned media from MSCs treated with TGF- α can induce blood vessel formation in an in vivo assay (De Luca et al. 2011). Aranha et al. have shown that MSCs cultured under hypoxic conditions showed copious secretion of pro-angiogenic factor VEGF in response to the activation hypoxia-inducible factor 1α (HIF-1 α)(Aranha et al. 2010). Preconditioning of MSCs has also been achieved by exposing the cells to intermittent cycles of ischemia and reperfusion. Repeated treatment with anoxia-reoxygenation activated HIF-1α signaling, activation of HIF-1α hypoxamiRs, downstream activation of survival signaling, and altering the paracrine profile of the preconditioned cells (Kim et al. 2009, 2012a). It has been observed that HIF-1α-dependent microRNAs are mechanistically involved in the preconditioning induced survival signaling (Haider et al. 2009). Given the significant role of HIF-1α in hypoxic and ischemic preconditioning and paracrine behavior of the cells, strategies have also been developed to activate HIF-1α through a non-hypoxic mechanism based on genetic modulation of the cells for concomitant overexpression of Akt and Ang-1 (Lai et al. 2012).

Besides hypoxia treatment, stem cells have also been preconditioned to support their rate of survival and modify their paracrine behavior (Haider and Muhammad Ashraf 2010). Some of the effective strategies in this regard include genetic modulation and pharmacological manipulation of the cells (Haider et al. 2008; Suzuki et al. 2010; Ahmed et al. 2010; Afzal et al. 2010). In addition, the production and secretion of pro-angiogenic factors by MSCs can also be enhanced by serum deprivation in the culture medium (Bianco et al. 2008). Furthermore, the conditioned medium from the cells cultured without serum could generate longer neovascular sprouts than the control cells-derived conditioned medium with serum during an ex vivo assay (Bianco et al. 2008). Put together, the secretion of the complex set of bioactive molecules in the secretome is susceptible to modulation through different approaches to promote angiogenesis.

Anti-apoptotic Activity

Several studies have demonstrated that MSCs can modulate cell apoptosis to restore the local microenvironment homeostasis in two different ways: direct cell-to-cell contact with the affected cell types and second through the secretion of paracrine mediators, such as IGF, FGF-2, HIF-1α, Heme oxygenase 1 (HO-1), and VEGF. Also, two different interleukins secreted by MSCs have a crucial role in the anti-apoptotic activity of MSCs-derived secretome on cells present in their microenvironment. For example, a recent study has shown that the anti-apoptotic activity of MSCs-derived secretome occurs mainly via IL10 activity since the abrogation of IL10 with a specific antibody added to the conditioned media significantly reduced the cytoprotective effects of the conditioned medium (Al-Azzawi et al. 2020). On the other hand, MSCs also inhibit lymphocytes and monocytes' apoptosis through an IL6 mediated mechanism (Xu et al. 2007; Raffaghello et al. 2008).

Overall, the paracrine activity of MSCs is key to avoiding caspase activation and subsequent apoptosis of endothelial, epithelial, and immune cells in a highly inflammatory microenvironment in the injured tissue.

Extracellular Vesicles

A variety of nano- and micro-sized vesicles, extracellular vesicles (EVs) shed from the cell surface as part of the paracrine activity of the cell, constitute the insoluble part of the secretome. According to the International Society for Extracellular vesicles (ISEV), the term EVs comprises the non-replicable small particles delimited by a lipid bilayer that do not contain a functional nuclei (Thery et al. 2018b). Initially, EVs release was thought to be a part of the mechanism in the cells aimed to eliminate unneeded cellular compounds; it is now clear that EVs are key players in intercellular communication. However, the EVs population is heterogeneous in size and shape, which has hindered our understanding of their features, molecular composition, and functionality. EVs are now classified, based on their size and biogenesis, into three major categories. Exosomes, Microvesicles, and Apoptotic bodies (Thery et al. 2018a).

Exosomes, generally 30–150 nm in diameter, are formed within the endosomal network and are released by the inward budding of multivesicular bodies (MVBs) (Raposo and Stoorvogel 2013, Yáñez-Mó et al. 2015). Alternatively, MVBs can be trafficked to lysosomes for degradation (Colombo et al. 2014). The fate of MVBs depends upon the cholesterol contents in their membrane, since cholesterol is necessary to avoid lysosomal degradation (Pfrieger and Vitale 2018). Microvesicles (MVs) biogenesis involves a less well-understood mechanism, however, it is generally perceived to involve outward budding of the extracellular membrane. MVs have a larger size, with a diameter ranging from 100 to 1000 nm. Because of their biogenesis, MVs are rich in proteins that are abundant in the plasma membrane, such as tetraspanins or proteins involved in cell-to-cell interaction through the recognizing of glycans and might facilitate the MVs-cell interaction. Finally, ApoBDs, released by apoptotic cells, are the largest EVs reaching up to 5000 nm in diameter. The first step in ApoBDs formation is the emergence of blebs in the plasma membrane that later become a protrusion, ending up in the release of the ApoBDs (Jiang et al. 2017). Interestingly, ApoBDs are considered essential players in the immune-modulated mechanism of implanted MSCs in the injured tissues (Galleu et al. 2017).

There are two different mechanisms by which EVs contribute toward the intercellular signaling. Firstly, EVs exert their effect upon uptake by the target cells, which involves the fusion of the vesicular membrane with the recipient cell membrane, thus enabling the release of EVs' cargo into the recipient cell cytoplasm. Alternatively, these vesicles could bind with the recipient cell membrane-bound receptors and activate specific downstream signaling pathways (Abels and Breakefield 2016).

Composition of EVs Cargo

Most of the pro-regenerative factors present in the MSCs' secretome are carried in EVs, mainly exosomes. This cargo is highly dynamic and reflects the parent cell lineage and its metabolic state. According to Exocarta, a manually curated standard

database of exosomes and their payload of proteins, RNAs, and lipids, there are currently registered 9769 proteins, 3408 mRNAs, 2838 miRNAs, and 1116 lipids identified in exosomes derived from various cell types. Importantly, different cell culture conditions and manipulations can greatly influence cargo loading in the exosomes, which might also impact their bioactivity. This can also be used for our own advantage by creating particular conditions that improve specific bioactivity of the exosomes produced by MSCs, making them more effective for a specific therapeutic application (Haider and Aramini 2019).

Although several analyses have been performed to establish a protein profile of EVs cargo' in different cell types, the diverging isolation and purification protocols have rendered this a daunting task and did not allow the production of conclusive data. Overall, the proteins present in the EVs cargo are proteins that are instrumental in their biogenesis, transmembrane proteins, different types of tetraspanins (CD9, CD63, and CD81), and proteins involved in antigen presentation (MHCI and MHC II). Although it is not as common, certain transcription factors have also been found as part of EVs cargo (Kalra et al. 2012).

As regards the lipid contents of EVs, they reflect their cells of origin, although specific lipids such as diacylglycerol and phosphatidylcholine seem to be less represented than the other membrane lipids. On the contrary, phosphatidylserine is more enriched in EVs compared to the cellular plasma membrane. Interestingly, it has been proposed that higher presence of phosphatidylserine in the membrane of some EVs facilitates their uptake by the recipient cells (Bicalho et al. 2013; Zaborowski et al. 2015).

Pertaining to the type of the genetic material found in the EVs cargo, it may include genomic and mitochondrial DNA and small RNAs of different types, that is, rRNA,tRNA, mRNAs, and lncRNAs. With a few exceptions that could correspond to either lncRNAs or intact mRNAs, the majority of RNAs found in the EVs cargo are less than 200 nucleotides in length. Among, the different nucleic acids found in the EVs cargo, miRNAs associated with exosomes are the ones that have been more intensively studied due to their capacity to alter the gene expression pattern of the target cell and their demonstrated role in different pathologies, including cancer (Asgarpour et al. 2020; Aramini et al. 2020). Generally speaking, the RNA cargo of the EVs mainly reflects the cytoplasmic contents of the cells of EVs' origin. However, some RNAs seem to be enriched in the EVs, thus suggesting the existence of some mechanism for cargo selection, such as the presence of particular sequences, named zip code, in the 3'UTR (Bolukbasi et al. 2012) on specific mRNAs or the "GGAG" motif found of several of the exosomal miRNAs (Villarroya-Beltri et al. 2013).

Currently, there is mounting evidence showing that the effect of the MSCs-derived EVs cargo on target cells is often mediated by the regulation of mitochondrial activity (Hogan et al. 2019). There is a significant increase in mitochondrial respiration and ATP production after exposure of the cells to MSCs derived EVs (Bland et al. 2018; Russell et al. 2019). In addition, several of the miRNAs, proteins, and mRNAs packed in the EVs have a direct or indirect effect on mitochondrial metabolism and baseline activity (Loussouarn et al. 2021). These organelles are

essential for cellular homeostasis, and thus, there are many pathologies related to mitochondrial dysfunction. Given the significant role of mitochondrial in cellular homeostasis, protocols are also being developed for mitochondrial preconditioning to support cell survival (Lu et al. 2010).

Applications of the Secretome from Mesenchymal Stem Cells in the Treatment of Musculoskeletal Diseases

A recent search on the clinical trials database (https://clinicaltrials.gov) for studies using MSCs secreted factors showed a total of 33 ongoing or completed trials. A quick screening of this list reveals that, although the number of studies involving MSCs secreted factors does not compare to the number of clinical studies involving MSCs, the therapeutic potential of MSCs-derived secretome is being tested in a wide variety of pathologies as a part of the fast-emerging cell-free therapy approach (Haider and Aslam 2018). In contrast to the low number of clinical trials using the cell-free therapy approach, there is a substantial number of preclinical experimental animal studies involving treatment with MSCs' secreted factors-containing conditioned medium. The encouraging data from the preclinical studies have generated immense interest in MSCs-based cell-free research to treat various injuries and pathologies. Interestingly, in the current pandemic scenario, the immunomodulatory and anti-inflammatory capacity of MSCs-derived exosomes is being tested in different clinical trials to treat the inflammatory symptoms associated with COVID-19 pneumonia.

MSCs secretome also shows therapeutic effect on the regeneration of the musculoskeletal system attributed to its multiple effects on the bone microenvironment, facilitating the communication between osteoblasts, osteoclasts, and bone marrow-derived MSCs (Liu et al. 2017). In this section, the role of MSCs secreted factors and EVs in bone, cartilage, and tendon regeneration would be discussed in detail.

MSCs Secretome for Bone Regeneration

Recent research is mainly focused on using MSCs secretome to treat bone-related pathologies encompassing from bone fractures to age-related changes, such as bone mass loss in osteoporosis, tissue degeneration of the osteonecrosis, etc. Systematic reviews have revealed that in almost all bone regeneration studies performed in various small animal models, treatment with MSCs-derived EVs has produced measurable benefits compared to controls. An outline of the different approaches tested in various animal models is shown in Fig. 2. Overall the therapeutic effects of EVs include a higher bone density, increased bone remodeling in osteoporotic models, less presence of necrotic tissue in osteonecrosis models with an increase in bone density, showing an increased number of proliferative cells and osteocytes, and finally, in rat fractures models, an enhanced formation of the callus (Tan et al. 2020).

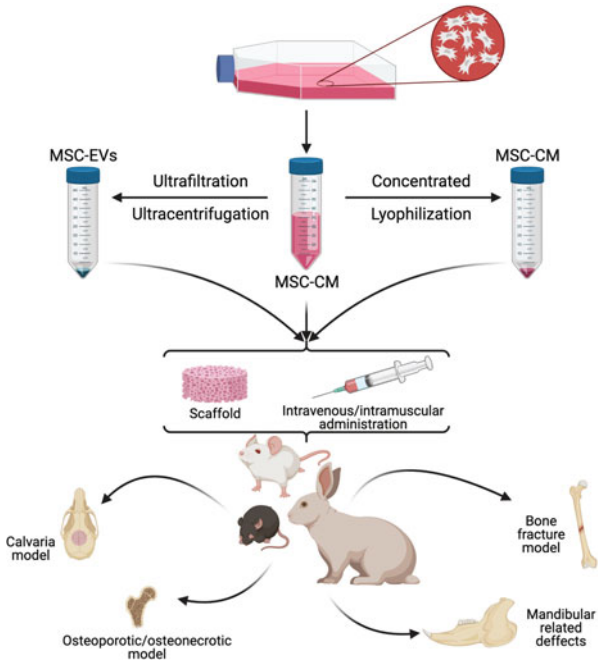

Fig. 2 Isolation, purification, and diverse applications of MSCs-derived EVs and conditioned médium on bone regeneration. (Created with **BioRender**.com)

Non-union bone defect after a long bone fracture is one of the main challenges in the orthopedic medical field due to the difficulties in achieving successful treatment. Current therapies are based on grafts and other bone substitutes; however, these are less effective for reconstructing bone defects that exceed a critical size (Roddy et al. 2018). These disadvantages underscore the need for searching novel therapeutic approaches that would allow the regeneration of these defects. The importance of MSCs-derived exosomes in critical-sized fracture repair was first assayed in CD9-/- mice, characterized by impaired fracture healing (Furuta et al. 2016).

In this experimental model, treatment with MSCs-derived exosomes rescued the fracture healing retardation observed in the control animals. The authors suggested that the reduced amount of MSCs-derived exosomes released in the CD9-/- mice were related to the healing deficiency.

Osteogenesis and angiogenesis are two highly related and intricate processes. The damage to local blood vessels during bone injury would impair bone regeneration. Therefore, the restoration of appropriate regional flow through neovascularization would ensure osteoinductive factors' access to the injured tissue, bone repair, and bone regeneration (Saran et al. 2014). Studies performed in an experimental rat model of femoral non-union clearly showed that MSCs-derived exosomes enhance osteogenesis and angiogenesis (Zhang et al. 2020a). Similar beneficial effects were observed in osteoporotic animals, indicating that MSCs-derived exosomes can also restore osteoporotic bone (Qi et al. 2016). Since osteogenic capacity is significantly reduced in osteoporotic MSCs, these effects could reflect an enhanced osteogenic

capacity of endogenous MSCs after exosomal treatment (Del Real et al. 2017). Mechanistically, MCP-1/-3 and IL-3/IL-6 in the conditioned medium have been shown to recruit bone marrow MSCs, mature endothelial cells, and progenitor cells to participate in angiogenesis and induce osteogenic differentiation (Ando et al. 2014).

As previously described, MSCs-derived exosomes can also benefit the age-associated systemic bone loss typical of osteoporosis (Zhao et al. 2018). However, the number of studies on this subject is scarce. MSCs-derived exosomes affect osteoblasts' activity through a SATB2 (special AT-rich sequence- binding protein 2) mediated mechanism (Yang et al. 2019). MALAT1, a lncRNA present in MSCs-derived exosomes, seems to act as a sponge for miR34c, inhibiting SATB2, a protein that is responsible for osteoblast activity (Yu et al. 2019). A common feature of all the studies involving treatment with MSCs secretome is the critical participation of miRNAs in mediating various pathological processes and regeneration of the bone tissue (Chen et al. 2019; Xu et al. 2020).

The combination of MSCs-derived conditioned medium, or EVs with different bioscaffolds, is an approach that has achieved optimal results in vitro and in vivo (Diomede et al. 2018a, b). The combination of exosomes derived from iPS-MSCs with tricalcium phosphate scaffolds has been shown to enhance bone regeneration by activating the PI3k/Akt signaling pathway (Zhang et al. 2016).

Leaving aside all the aspects that require in-depth studies, MSCs-derived exosomes have already been tested in humans in combination with specific scaffolds. For example, a case-control study used exosomes for alveolar bone regeneration, describing a denser bone formation, lower inflammation, and no abnormal delayed healing (Katagiri et al. 2016). A preclinical trial in humans has also been performed with promising results (Katagiri et al. 2017). Although much work needs to be done in this field, these data constitute the lead of future studies.

MSCs Secretome in the Treatment of Cartilage Defects and Cartilage Degeneration

Articular cartilage defects in weight-bearing joints represent one of the major pathologies of the musculoskeletal system. The etiology of these defects is multifactorial, but the most prevalent pathologies related to articular cartilage are primary osteoarthritis (OA) and posttraumatic cartilage degeneration. OA is a degenerative disease characterized by the destruction of articular cartilage and synovial inflammation (Musumeci et al. 2015){Musumeci, 2015 #773}. Regardless of the etiology, the deficient intrinsic cartilage self-repairing ability is the main issue when dealing with cartilage damaging diseases (D'Arrigo et al. 2019). Classical contemporary treatment for OA is only symptomatic and is aimed to alleviate pain and inflammation without successfully containing the vicious process of cartilage degeneration. When these treatments are no longer effective, joint replacement is the only possible way of action. To avoid surgery that could lead to long-term complications, more treatment options are currently being studied. Current surgical interventions include microfractures, mosaicplasty, perforations and abrasions, or the transplantation of autologous chondrocytes (ACI and MACI techniques) that fail to prevent the

progression of the disease over the long term or to provide long-lasting symptomatic relief (Lee and Wang 2017). Importantly, OA incidence is rapidly increasing due to the global aging of the population and the obesity epidemic, urging new efficient therapies to promote cartilage regeneration.

The intra-articular injection of BM-MSCs or adipose tissue-derived MSCs (A-MSCs) directly into the affected joint has significant anti-inflammatory and anti-catabolic effects besides pain relief and improved functional scores (Yokota et al. 2019). These beneficial effects have been attributed to the MSCs' paracrine capacity (Mancuso et al. 2019). It has been estimated that paracrine activity is responsible for at least 80% of the MSCs favorable effects on cartilage regeneration (Muhammad et al. 2019). However, this approach shares the limitations of other MSCs-based therapies. Besides, cell-based therapies for the treatment of OA are subjected to the same problems already mentioned in the introduction of this work.

Most of the current studies related to the role of the secretome in OA treatment focus on the insoluble fraction of this secretome, particularly in the exosomes. To date, MSCs-derived exosomes attenuate cartilage degeneration in different experimental animal models of OA (Ni et al. 2020). Furthermore, compared with MSCs transplantation, exosomes-based treatments exhibit higher stability and flexibility and a wide range of immunomodulatory activity.

MSCs-derived exosomes have demonstrated their ability to promote regeneration and repair of both cartilage and subchondral bone (Asghar et al. 2020). Exosomes can also prevent osteoarthritic chondrocytes from undergoing apoptosis via fusion with chondrocyte mitochondria, a process that alleviates the mitochondrial dysfunction generally associated with degenerative chondrocytes (Qi et al. 2019). Some of the exosomal cargo moieties with a pivotal role in cartilage regeneration have already been identified. Mao et al. (Mao et al. 2017) found that miR-92a-3p overexpressing BM-MSCs-derived exosomes were able to promote chondrogenesis, prevent cartilage matrix degradation, and delay OA progression in an experimental model of OA. As previously discussed, MSCs-derived EVs are responsible for a wide range of immunoregulatory activities during the cartilage regeneration process. The initial stages of OA are linked to an increase in the synthesis of pro-inflammatory cytokines, such as ILβ or TNFα, which stimulates the expression of multiple cytokines and Ptgs2, one of the most potent pro-inflammatory genes (Sandell et al. 2008). A reduction in Ptsg2 expression in OA can be achieved by treatment with MSCs-derived exosomes overexpressing micro-RNA-26a-5p. This leads to an overall reduction in the pro-inflammatory factors (Jin et al. 2020).

MSCs have been modified by drug preconditioning or gene modification to produce exosomes with a specific payload that could improve cartilage repair. Preconditioning of BM-MSCs with TGFβ3 increases the expression of anabolic markers and decreased levels of catabolic marker genes in OA chondrocytes (Cosenza et al. 2017) and enhances the biomechanical characteristics of engineered cartilage (Byers et al. 2008). On the other hand, preconditioning of BM-MSCs with small-molecule Kartogening produced exosomes that promoted the formation of a more robust chondral matrix through the regulation of the metabolic activity of chondrocytes (Liu et al. 2020a){Liu, 2020 #787}.

Besides BM-MSCs, MSCs from other tissue sources have also been studied for their therapeutic potential in OA. Synovial MSCs (S-MSCs) showed chondrogenic differentiation and cartilage promotion and attenuated OA in experimental animal studies (Enomoto et al. 2020; Kondo et al. 2019). Exosomes derived from A-MSCs decreased inflammation, promoted cartilage regeneration, enhanced the proliferation and migration of human OA chondrocytes, and successfully maintained the chondrocyte matrix (Damia et al. 2018; Tofino-Vian et al. 2018). MiR-100-5p, found in A-MSCs derived exosomes, decreased the severity of cartilage lesions and improved gait in the experimental mice model (Wu et al. 2019). On the other hand, human A-MSCs-derived exosomes had down-regulated pro-inflammatory genes, enhancing proliferation and chondrogenic potential of periosteal cells via upregulation of miR145 and miR221 (Zhao et al. 2020).

Put together, these data reinforce the essential role of exosomes to prevent and treat OA in the future. However, more preclinical experimental studies are needed, especially to know their role in long-term OA evolution. To our knowledge, there is no clinical study evaluating exosomes' role in the treatment of human OA patients.

MSCs Secretome in the Treatment of Tendon Injuries

Tendon injuries, both chronic and acute, are prevalent debilitating diseases. Tendon has a limited inherent self-healing capacity. Either conservative or surgical treatment results in a far from satisfactory prognosis. The situation is further aggravated due to a high prevalence of early re-rupture and tissue scarring tissues in the lesion field (Lui 2020). Cell-based therapy of injured tendons using MSCs promotes tendon repair (Hevesi et al. 2019), although these therapeutic effects are mainly executed by paracrine factors (Connor et al. 2019).

Transplantation of rat BM-MSCs derived exosomes, seeded on fibrin glue, into a patellar tendon wound resulted in superior tendon healing (Shi et al. 2019). At the microscopical level, the treated tendons showed better-aligned collagen fibers and better biomechanical results (Yu et al. 2020). Interestingly, this improvement was dose-dependent, with tendons treated with a high dose of exosomes achieving better histological tendon-fiber alignment and vascularity (Gissi et al. 2020). Besides fibrin glue, other carriers have been used in these studies. For example, EVs derived from A-MSCs loaded onto a collagen sheet showed decreased postoperative gap formation and re-rupture on a rat Achilles tendon model (Shen et al. 2020). In addition, collagen formation at the injury site was also facilitated and early inflammatory response after the lesion was abrogated.

The aforementioned studies are based on the direct action of MSCs-derived exosomes on tendon healing; however, indirect applications of these MSC-derived EVs have also been tested. Macrophages pre-treated with MSCs-derived exosomes promoted tendon healing, a reduction in inflammation, and an improvement in the biomechanical properties when applied to an experimental murine Achilles tendon model (Chamberlain et al. 2019).

Although there are registered clinical trials for therapeutic assessment of EVs, to our knowledge none of these trials is on tendon healing. Most of the research done in this area corresponds to preclinical experimental studies mainly focused on acute lesions. Future studies are therefore warranted for their therapeutic assessment in the long-term and chronic settings. Since the therapeutic benefits are dose-dependent, it is important to improve our knowledge on the exosome concentration in dose-escalation studies to determine optimal exosomal dose. On the same note, the timing of injection after injury and the frequency of exosomal treatment need to be optimally defined.

Modulating Secretome Therapeutic Properties

One advantage of MSCs' secretome for therapeutic application is that the composition of the secretome as well as that of exosomal cargo can be modulated to fit the specific treatment requirements of the damaged tissue. Consequently, various approaches have been developed to tailor MSCs-derived secretome and its exosomal payload for specific clinical applications. The following section focuses on the conditions and methods designed to enhance the therapeutic efficacy of MSCs secretome for bone, cartilage, and tendon regeneration therapies (Chang et al. 2021). A summary of these methods is shown in Fig. 3.

Hypoxia Induction

While standard cell culture is carried out in normoxia (21% O_2), oxygen tensions ranging from 0% to 10% are often used to mimic the conditions found in MSCs niches for cell expansion in vitro. Therefore, it has been generally considered that MSCs cultured under hypoxic conditions simulating the in vivo MSCs niche microenvironment would help the cells sustain their biological characteristics and full therapeutic potential (Ferreira et al. 2018). Indeed, hypoxic preconditioning seems to potentiate MSCs' regenerative and cytoprotective effects and bioactive factors secretion in general (Ferreira et al. 2018). MSCs' secretome produced under hypoxic conditions is rich in proangiogenic factors, such as VEGF, TGF-β1, angiogenin (Agn), or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Quade et al. 2020).

Moreover, other important therapeutic properties such as more vigorous immunomodulatory activity (Costa et al. 2021), enhanced chemotactic attraction to the chemical cues, that is, SDF-1 and monocyte chemoattractant protein-1 (MCP-1) (Quade et al. 2020), superior anti-apoptotic, anti-oxidative stress, and anti-fibrotic functions, have also been attributed to MSCs secretome generated under low-oxygen culture conditions (Collino et al. 2019; Miceli et al. 2021). In fact, hypoxia is the most commonly used priming method for enhancing the production of a secretome with greater regenerative potential. However, there are still some issues to overcome before these parameters can be standardized.

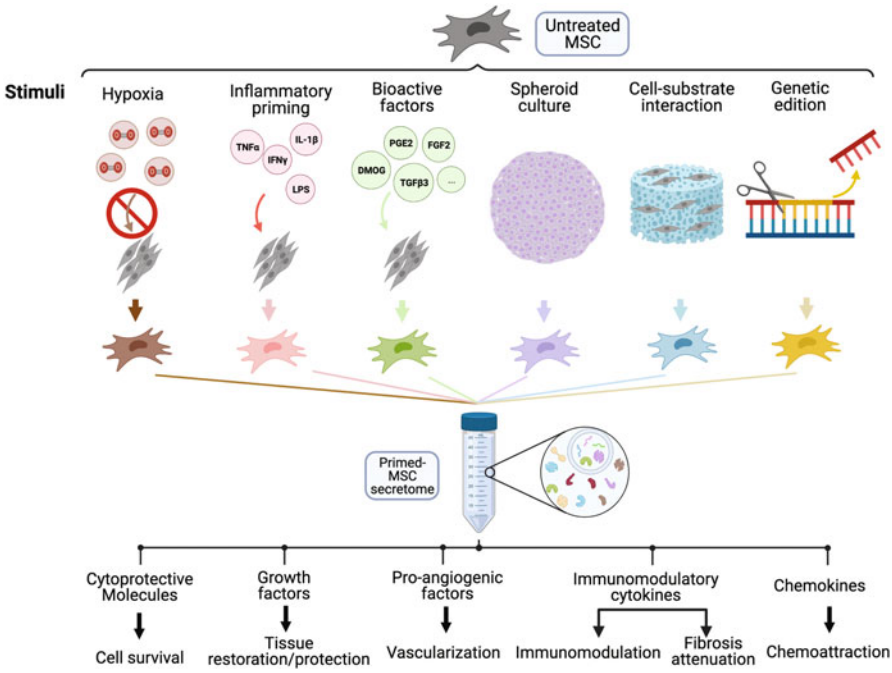

Fig. 3 The commonly used MSCs' secretome modulation methods to enhance their therapeutic properties. Hypoxia, inflammatory factor priming, induction of bioactive factors, spheroid culture, cell-substrate interaction, and genetic edition are the most well-studied stimuli that have been reported to treat different pathologies using primed secretome. (Created with **BioRender**.com)

For instance, a wide range of O_2 concentrations has been used to precondition the cells. Also, secretome composition is substantially modified depending on the duration of the cells exposure to hypoxia (Quade et al. 2020). Efforts are underway to establish an optimal hypoxic priming protocol that maximizes the therapeutic characteristics of MSCs' secretome (Costa et al. 2021).

Inflammatory Priming

Activation of MSCs to repair damaged tissue is triggered by inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α (Miceli et al. 2021). Pro-inflammatory stimulus in the culture medium is one of the many available methods to modulate MSCs' secretome that has received more attention. The three cytokines mentioned above, together with lipopolysaccharide (LPS), are the main effectors used in vitro for simulating the inflammatory microenvironment that elicits the secretion of immunomodulatory factors by MSCs.

This priming protocol produces results that encompass, complement system inhibition, inhibition of NK cells, the guidance of monocyte differentiation to M2

anti-inflammatory phenotype, suppression of cytotoxic T cell proliferation, inhibition of dendritic cell maturation, and an increase in regulatory T cells (Treg). Increased prostaglandin E2 (PGE2), indoleamine-2,3-dioxygenase (IDO), cyclooxygenase 2 (COX-2), and TGF-β are responsible for these effects (Costa et al. 2021; Ferreira et al. 2018). IL-6, the primary inducer of inflammation, is also overexpressed in inflammatory factor-primed MSCs' secretome, but its concentration decreases over time after priming (Bundgaard et al. 2020). Although this seems to be contradictory, a correct regeneration process initially requires an acute inflammatory phase to trigger the influx of immune cells and homing of MSCs to the injury site that would later participate in the repair process (Bogatcheva and Coleman 2019). Preconditioned MSCs' secretome facilitates this process as it maintains primary inflammation long enough to trigger subsequent angiogenesis, and then exerts anti-inflammatory effects. Subsequently, a complex remodeling process sets, which is marked by an increase in matrix metalloproteinase 1 and 3 (MMP1 and MMP3), metalloproteinase inhibitor 1 precursor (TIMP1), and plasminogen activator inhibitor 1 (PAI-1) (Maffioli et al. 2017; Bundgaard et al. 2020).

Inflammatory factor-primed MSCs' secretome also performs an essential pro-trophic action. For example, it has been reported that INF-γ-preconditioned MSCs' secretome promoted tendon healing and chondroprotection (Shen et al. 2020; Maumus et al. 2016; Ragni et al. 2020) while TNF-α priming exerts bone regenerative function (Lu et al. 2017).

Although these inflammatory stimuli are similar, many studies have shown diverse effects of MSCs' priming with pro-inflammatory cytokines as IFNγ, TNF-α, or IL-1β (Ragni et al. 2020). Therefore, further refinement of the protocols in terms of the concentration of cytokines, time of induction, cytokine combination, etc., is required to obtain the optimal MSCs' secretome composition for optimal prognosis.

Addition of Bioactive Factors

MSCs can also be primed by adding soluble bioactive molecules to the culture media during cell culture and expansion. For example, given the benefits of hypoxia priming, treatment of the cells with dimethyloxaloylglycine (DMOG), a small molecule that stabilizes HIF-1α, or deferoxamine (DFO), an iron-chelating agent, may simulate hypoxic culture conditions without exposure to hypoxia. Both hypoxia mimicking agents increase angiogenic growth factor expression in MSCs. In particular, DMOG preconditioning has been shown to significantly induce bone regeneration (Chang et al. 2021). On the same note, Vadadustat, a pharmacological hypoxia inducer, has been used to precondition MSCs. Preconditioning MSCs with Vadadustat has led to an augmented anti-inflammatory effect with a concomitant increase in angiogenic growth factors expression and secretion (Zielniok et al. 2020).

Besides hypoxia mimicking agents, FGF2, resveratrol, omentin-1, or thrombin have been studied to prime MSCs, leading to an enhanced angiogenic potential of the cells (Liu et al. 2020b; Sung et al. 2019). Moreover, priming with thrombin contributes to increased EV production and alleviates inflammation. Furthermore, it

was revealed that anti-inflammatory MSCs secretome could be obtained by priming the cells with valproic acid, melatonin, PGE2, substance P, or tetrandrine. Interestingly, tetrandrine was selected and validated from a group of 1402 FDA-approved drugs using a high throughput screening protocol and showed potent immunosuppressive effects (Cabezas et al. 2020; Chang et al. 2021; Heo et al. 2020).

Besides, it was stated that secretome from MSCs primed with TGFβ3, hyaluronic acid, or curcumin showed chondroprotection and improved osteoarthritis (Ruiz et al. 2020; Zhang et al. 2021). Similarly, Kim et al. reported that pioglitazone-primed MSCs' secretome induced cell proliferation and secretion of soluble collagen in tenocytes, leading to tendon regeneration. Interestingly, increased VEGF contents in pioglitazone-primed MSCs' secretome had a role in tendon remodeling despite poor vascularity (Kim et al. 2019).

Modulation of Cell-Cell Interactions

Although two-dimensional (2D) culture is a standard technique for culturing MSCs in vitro; this setting is artificial compared to physiological conditions. Furthermore, 2D culture provokes loss of crucial receptors, increases the expression of pro-inflammatory chemokines, and is highly deficient in cell-cell interactions, thus causing reduced MSCs stemness (Costa et al. 2021; Chang et al. 2021).

On the contrary, the formation of 3D structures, known as MSCs spheroids, provokes changes in cell morphology, cytoskeletal organization, intracellular signaling pathways, and gene expression (Ferreira et al. 2018). In addition, this type of culture aids cell-to-cell and cell-to-ECM interactions. It also contributes to a microenvironment that simulates the natural habitat of the cells in their niches, wherein the inner layer cells are exposed to low hypoxia and nutrients (Chang et al. 2021). These subtle but influential adjustments are directly responsible for enriching immunomodulatory, angiogenic, and prosurvival factors in 3D spheroid-cultured MSCs' secretome (Chang et al. 2021). Moreover, the secretome would be rich and in exosomal contents (Kim et al. 2018a). On the one hand, due to hypoxia, MSCs located in the spheroid core overexpress angiogenic factors through a HIF-1α and HIF-2α-mediated mechanism. On the other hand, spheroid conformation reduces mitochondrial membrane potential and ATP production, indicating apoptotic processes in MSCs, which lead to IL-1 release. Then, self-induction by autocrine effects of IL-1 enhanced the anti-inflammatory profile of MSCs' secretome, which closely relates to cell survival and anti-fibrotic factors (Cesarz and Tamama 2016).

As of today, a few studies corroborate the beneficial effects of the secretome derived from spheroids cultured MSCs (Santos et al. 2015; Miranda et al. 2019). Apart from these scarce published reports, the alleged beneficial effects of their secretome are generally based on the presence of special components rather than any direct evidence. Importantly, spheroid-priming due to 3D culturing seems to have a synergistic role when combined with other forms of priming, that is, inflammatory stimuli (Redondo-Castro et al. 2018), MSCs' encapsulation, or the use of microcarriers (Saldana et al. 2019; Tsai et al. 2020).

There are currently several technical details, such as spheroid induction protocols, optimal cell size, cell density, etc., that need to be defined to standardize the production for the maximal therapeutic potential of their secretome (Costa et al. 2021).

Tuning Cell-Substrate Interaction (ECM and Scaffolds)

Another protocol of 3D priming is based on using scaffolds that support, or rather enhance, cell-substrate interaction. Since extracellular signals are crucial for cell adhesion, organization, migration, and growth, this approach of substrate-culture preconditioning can play an essential role in MSCs secretome composition.

Thomas et al. studied the changes in MSCs secretome when those cells were cultivated in collagen microgel and demonstrated that proper cell density, collagen-crosslinker ratio, and collagen concentration could substantially increase proangiogenic and immunomodulatory cytokine concentration in MSCs secretome (Thomas et al. 2014). Furthermore, due to the mechanosensitive nature of MSCs, the increased stiffness of polyacrylamide hydrogels functionalized with fibronectin gets translated into higher cell spreading and a more robust cytoskeleton. These molecular and cell structural changes result in proangiogenic growth factor-rich MSCs secretome with significantly higher tubulogenic potential in vitro (Abdeen et al. 2014). On the same note, exposure of MSCs to direct mechanical load significantly alters their secretome profile favoring chondrogenic and angiogenic induction (Gardner et al. 2016; Kasper et al. 2007). The topography of the material supporting MSCs growth also significantly affect the secretome composition. Leuning et al. produced 76 different algorithm-generated well-plate surfaces and displayed that each one provoked a unique cytokine secretion profile (Leuning et al. 2018). On the same note, substrate porosity is relevant to cell culture characteristics and hence, has a significant bearing on their secretome profile. MSCs grown on macroporous scaffold show a substantial enrichment in cytokines related to tissue regeneration in comparison to nanoporous hydrogel substrate, even though both exhibit same stiffness. These changes in secretome profile have been attributed to the increase of N-cadherin mediated cell-cell interactions in the macroporous scaffold, which correlates well with the aforementioned spheroid culture impact on MSCs' paracrine secretions (Qazi et al. 2017). Apart from this, it is imperative to highlight the use of decellularized ECMs as a scaffold to show positive effects on the proangiogenic profile of MSCs' secretome (Sears and Ghosh 2020).

Therefore, different substrate properties can be combined to enrich MSCs' secretome profile toward a certain cluster of factors of interest. Likewise, a combination of cell-substrate tuning with other types of preconditioning, such as inflammatory priming, was reported to result in a synergistic effect (Wong et al. 2020). Deconstructing the physical and biochemical cues implied in cell-substrate primed MSCs' secretome will help to design optimal platforms for induction of a desired therapeutic effect (Abdeen et al. 2014).

Genetic Editing

A genetic edition is a solid tool that has been used to genetically modulate MSCs' ability for long-term transgene expression and to modify their paracrine behavior (Haider et al. 2008). However, in the majority of cases, the application of gene-editing was aimed at improving cellular characteristics such as survival, proliferation, or differentiation capacity and with the aim of using MSCs as vehicles for the delivery of certain molecules (Jiang et al. 2006; Ren et al. 2008; Fierro et al. 2011; Costa et al. 2021).

From the standpoint of cell-free strategies, some studies revealed that RUNX2 and BMP2 overexpression in MSCs lead to MSC-EVs with higher osteogenic commitment (Martins et al. 2016; Huang et al. 2020). Similarly, simulating hypoxia conditions through HIF-1α overexpression in MSCs, led to the production of angiogenic exosomes with enhanced pro-osteogenic and pro-angiogenic activities (Li et al. 2017). The genetic modification approach has also been used to reprogram MSCs to pluripotency for use as induced pluripotent stem cells (Buccini et al. 2012).

Although genetic modification has been associated with tumorigenic risk, the use of cell-free therapies allows to open a new set of possibilities since modified cells are not directly applied to patients. In any case, more research is needed to characterize genetically-modified MSCs' secretome. This is a crucial step before this strategy could be applied in clinical trials.

Disease-Like Priming

In vitro mimicking MSCs' specific natural niche microenvironment in certain pathologies may produce a tailor-made secretome that could be extremely useful in the treatment of these pathologies. An example of this priming method is outlined in Fig. 4**.** Recent studies have attempted to demonstrate this hypothesis. Cifù et al. have shown that MSCs exposed to osteoarthritic synovial fluid increased the release of immunosuppressive factors (Cifu et al. 2020). Moreover, a test applying healthy, traumatic, and degenerative human intervertebral disc cells (IVDs)-derived conditioned medium to MSCs showed that the profile of the produced secretome was highly dependent on the IVDs cells' status. Moreover, the protein composition enhanced homeostasis maintenance in the case of healthy IVDs and damage repair in the case of traumatic and degenerative IVDs. Even differences between the traumatic and degenerative priming were also observed (Wangler et al. 2021). Importantly, MSCs primed with serum of the patients that have suffered poly-traumatism showed a secretory profile that could not be simulated by induction with a selected cocktail of trauma-relevant factors (Amann et al. 2019). These data showed that the serum of the patients might have other factors that would activate a slightly different set of genes.

Although disease-like priming seems to be highly promising, it remains to be seen whether this approach may be applied to any type of disease. A way forward could be

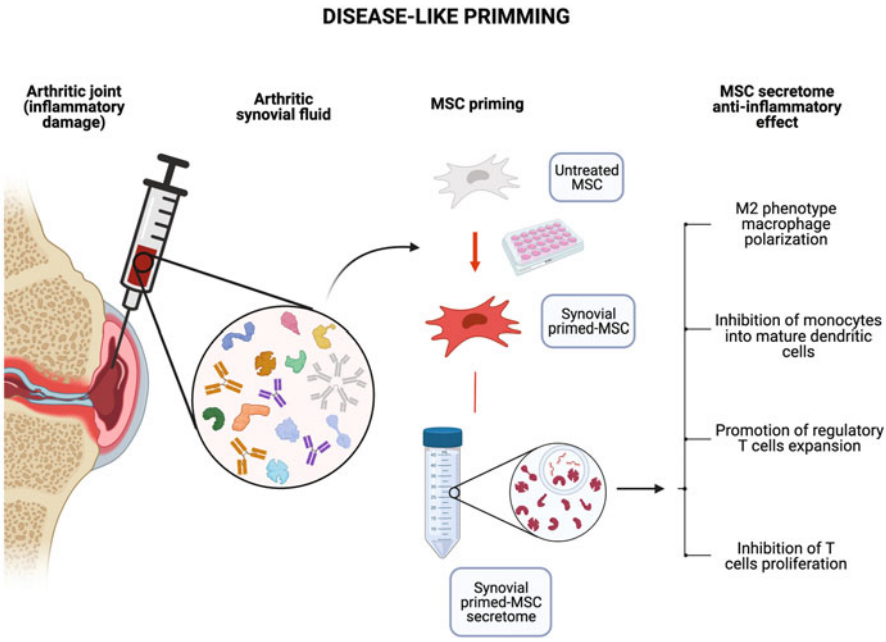

Fig. 4 Osteoarthritic-like priming based on the work by Cifu et al. (2020). OA-primed MSC secretome demonstrated the capacity to acquire functions that support the resolution of osteoarthritic pathology. (Created with **BioRender**.com)

the emergence of artificial tissue-engineered organs to model diseases in vitro. Once the standardization issues applicable to all the secretome-based techniques are solved, secretome preparations tailored to specific clinical applications may be developed. According to the current tendency for medical treatments, this strategy will also presumably progress toward the so-called personalized medicine.

Future Perspectives

MSCs have a significant and recognized role in tissue regeneration. Hundreds of patients have been treated in controlled clinical settings with promising results. However, translation to the clinic is still experiencing many obstacles. According to the regulatory agencies, convincing evidence of safety, especially MSCs from sources other than bone marrow, is lacking (Marks et al. 2017; Mendicino et al. 2014). Also, many clinical protocols were based on the idea that MSCs will always meet damaged tissue needs in what can be called the "MSCs knowing what to do" way (Pittenger et al. 2019). The published clinical data analysis has shown that around 50% of patients do not respond to treatment (Caplan 2018). It remains to be ascertained whether this high number of non-responders is due to differences in cell donors, cell preparation protocols, the disease stage at which the treatment is applied,

or even the patients' genetics. To make MSCs' cultures suitable for clinical use, an in-depth research is needed to identify and define all these variables.

During the last decade, the role of the paracrine activity of MSCs in tissue regeneration has been highlighted to explain the therapeutic benefits of cell therapy. A head-to-head proteomic profile of MSCs' secretome with various other cells has shown that BM-MSCs produced larger amounts of secretome proteins (Billing et al. 2016). This is consistent with the greater importance of the secretome in MSCs biology and makes MSCs the cells of choice for clinical applications.

MSCs' secretome has some practical advantages over MSCs themselves. First are the logistic advantages that allow ease of storage, handling, and off-the-shelf availability. Second, its administration does not require cell-culture-trained personnel.

Third, regarding safety, as it does not have a cellular component, secretome is less immunogenic, and the risk of malignancy is reduced. As with cell cultures, a great effort is needed to standardize secretome contents, and the RNA Communication Consortium (ERCC1) (Das et al. 2019) and the ISEV (Thery et al. 2018b) have released guidelines to assist researchers accordingly.

Clinical application of the secretome also presents many of the difficulties that affect cell-based applications. The lack of standardization of MSCs cultures is a significant problem in translating it to the clinic, while the origin of MSCs is an essential determinant of variability. A comprehensive study using both RNAseq and quantitative proteomics comparing commercial BM-MSCs and Embryonic stem cell-derived MSCs (ESC-MSCs) has reported 2500 differentially expressed RNAs and, depending on the stringency, 40–200 differentially expressed proteins in the two cell types. Most interestingly, MSCs from young donors have a profile closer to ESC-MSCS, which indicates that MSCs gene expression changes with age (Billing et al. 2016). Furthermore, depending on their origin, MSCs secrete different profiles of paracrine factors (Hsiao et al. 2012), which correlates well with various therapeutic capacities. As an example, A-MSCs have less osteogenic potential than BM-MSCs (Niemeyer et al. 2010), but in contrast, A-MSCs produce better results than BM-MSCs for ischemic stroke therapy (Ikegame et al. 2011). Likewise, the proteomic profile of MSCs' secretome isolated from three tissue sources identified a total of 1533 proteins, of which only 124 were shared between the three tissue samples (Tachida et al. 2015). These data were confirmed in subsequent studies comparing MSCs' secretomes from different tissues (Shin et al. 2021), and they possessed different therapeutic abilities (Daneshmandi et al. 2020).

When it comes to the secretome, standardization is hampered by the limited expansion capacity of the primary cultures and the need to constantly replenish cell sources. Immortalization of cells could be a valid alternative to achieve an unlimited supply of cells with similar characteristics. It has been reported that MYC-transformed MSCs yield exosomes in the milligram range. While the cells' adipocytic differentiation was affected, the exosomes maintain the ability to reduce relative infarct size in a mouse model of myocardial ischemia /reperfusion (Chen et al. 2011). *Another immortal line*, constructed by hTERT overexpression, maintained most primary cells' characteristics, including immunosuppression (Wolbank et al. 2009). Compared to the possible specialization resulting from

adapting the cells' origin to each of the different pathologies mentioned above, using a single cell line may be counterproductive. Nevertheless, the fact that culture conditions determine the secretome's properties could compensate for this disadvantage.

Changes in culture conditions may be used to adapt secretomes to particular therapeutic needs. For example, hypoxic culturing protocols maintain MSCs' multipotency and cell proliferation (Hawkins et al. 2013). The secretome of TNF-α-activated MSCs showed improved wound healing properties, while 3D culture MSCs in spheroids had better pro-angiogenic potential (Xu et al. 2016). Many other changes in culture conditions, including hydrogels (Qazi et al. 2017), cytokines, hormones, growth factors, and drugs like atorvastatin, are being used to introduce changes in the cells' secretome profile (Ferreira et al. 2018). The specific products and the precise mechanisms by which the microenvironment modulates the secretome profile are undefined. Still, the path is now open to understanding the conditions via in-depth research (Kusuma et al. 2017).

EVs-related research is receiving the most interest from researchers. Not only because of their biological properties but also due to their inherent cargo of bioactive molecules, including RNAs and proteins, which they deliver to the recipient cells. They provide a basis to build nano-sized vehicles for the delivery of pharmaceutically relevant substances. Historically speaking, tumor cells are the primary source of EVs for pharmaceutical applications. Still, MSCs produce a large amount of secretome (Billing et al. 2016), and coupled with potentially lower tumorigenicity, make MSCs a safer source of EVs for drug development (Villa et al. 2019). Unmodified EVs reach high blood concentrations soon after administration, but their clearance rate is very high (Wiklander et al. 2015). In vitro modification of the particles by conjugates of glycerol-phospholipid-polyethene glycol prevents their elimination by the endothelial reticulum system. This procedure, known as cloaking, also allows specific ligands to be attached to the particle to provide tissue specificity. The cloning and transfection of DNA encoding hybrid proteins that alter the membrane's outer properties, called surface display, is an alternative that is also receiving much attention (Antes et al. 2018).

Cell culture in the presence of drugs is a simple method to produce anti-tumor-enriched EVs (Goh et al. 2017), but the disadvantage is that the drug itself can lead to toxicity in the cultures. On the other hand, the genetic modification of cells in culture is more efficient using plasmids encoding for miRNA precursors or directly with miRNA mimics. The resultant EVs, mostly exosomes, are enriched in specific miRNAs (Kim et al. 2018b).

EVs can also be modified for site-directed delivery to the target tissue. This is usually achieved by adding either small peptides or antibodies which recognize receptors present on the target cell (Murphy et al. 2019). Because of the ease with which they are incorporated into particles, aptamers, and small nucleic acid molecules functionally similar to antibodies are becoming a valuable alternative for tissue-specific delivery of EVs (Zou et al. 2019).

In conclusion, the secretome, a highly complex mixture of proteins, lipids, RNAs, and EVs, mimic the therapeutic properties of MSCs cultures. The secretome

presents several practical advantages that could make it a valid alternative to some MSCs culture applications. It has sometimes been possible to attribute the benefits of the secretome to specific proteins or RNAs. Furthermore, the application of constituent molecules of the secretome in isolation is also possible. However, in most preclinical studies, the molecules responsible are unknown and the effects observed and reported are the outcome of several constituent molecules present in the secretome. Much more information is still needed to enable us to transfer the knowledge to the clinic with confidence. However, combined with a more profound understanding of the ideal culture conditions, the availability of immortalized cell lines would help to develop homogenous product batches suitable for treating specific pathologies. Finally, it is pertinent to mention that EVs, with minor modifications, are ideal vehicles for targeted drug delivery. The knowledge of MSCs biology combined with various biomaterials and pharmaceutical technology offers endless possibilities for developing innovative and smart therapies based on MSCs' secretomes.

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Part IV

Miscellaneous Aspects of Stem Cell Applications

Common Ethical Considerations of Human-Induced Pluripotent Stem Cell Research

39

Adekunle Ebenezer Omole, Adegbenro Omotuyi John Fakoya, Kinglsey Chinonyerem Nnawuba, and Khawaja Husnain Haider

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Abstract

In 2006, Shinya Yamanaka generated induced pluripotent stem cells (iPSCs), which has been the major scientific event of the decade that caught the eye of many scientists, politicians, and bioethicists. The use of human embryonic stem cells (hESCs) has previously been limited by ethical issues related to the

A. E. Omole
Department of Anatomical Sciences, American University of Antigua College of Medicine, St. John's, Antigua and Barbuda
e-mail: kunlesty@yahoo.com

A. O. J. Fakoya (✉)
Department of Anatomical Sciences, University of Medicine and Health Sciences, Basseterre, Saint Kitts and Nevis
e-mail: gbenrofakoya@gmail.com

K. C. Nnawuba
Department of Clinical Medicine, School of Medicine, Caribbean Medical University, Willemstad, Curaçao
e-mail: knnawuba@yahoo.com

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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destruction of embryos. However, with iPSCs, scientists can now reprogram virtually any human somatic cells through the expression of a combination of embryonic transcription factors to a pluripotent embryonic stem-cell-like state, thereby avoiding the contentious destruction of human embryos. Although the clinical realities of human-induced pluripotent stem cells (hiPSCs) appear very promising, they are still laden with some ethical concerns that scientists and legal authorities in the field of iPSC research must recognize. This chapter briefly reviews some ethical issues associated with the use of hiPSCs and suggests ways to address these challenges.

Keywords

Ethics · Human-induced pluripotent stem cells · Human embryonic stem cells · Moral issue · Patenting · Reproduction · Informed consent

Abbreviations

CNV	Copy number variations
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
hESCs	Human embryonic stem cells
HFEA	Human Fertilization and Embryology Authorities
hiPSCs	Human-induced pluripotent stem cells
iPSCs	Induced pluripotent stem cells
IVF	In vitro fertilization
MACS	Magnetic-activated cell sorting
SCNT	Somatic cell nuclear transfer
SNV	Single nucleotide variation

Introduction

For decades, ethical debates regarding stem cell technology have focused mainly on human embryonic stem cells (hESCs). These cells are harvested from the inner cell mass of blastocysts (preimplantation embryos) and obtained with consent from couples receiving in vitro fertilization (IVF) treatment, from aborted fetuses, or from donated oocytes (Thomson et al. 1998; Smith 2001; Zhang et al. 2006). The embryonic origin of hESCs raises a mix of serious moral and ethical controversies about the onset of human personhood, treatment, and harm to embryos; concerns about the safety and health risks of women donating eggs, the potential exploitation of their ova, and their informed consent; and concerns about respect for human life, human dignity, and justice toward humankind. These ethical debates reveal deeply rooted individually diverging opinions about the nature and origin of human personhood, leading to differing policies and regulations of hESC research worldwide (De Trizio and Brennan 2004; Solo and Pressberg 2007; Dhar and Hsi-En Ho 2009). Furthermore, due to this diversity of opinions and cultural differences, an

international consensus regarding the regulation of hESC research does not exist (Dhar and Hsi-En Ho 2009). The resulting restrictions and prohibitions on hESC research have contributed largely to the slowness in the progress on the translation of hESC technology into clinical therapy. Hence, there was an urgent need for another substitute for hESCs with the same pluripotency potential that can bypass these ethical issues.

Shinya Yamanaka's 2006 discovery of induced pluripotent stem cells (iPSCs) was a notable breakthrough in stem cell research, which has given it a new impetus (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Omole and Fakoya 2018). Scientists and bioethicists were excited at the ability to fabricate a surrogate cell with a pluripotent embryonic stem cell (ESC)-like state by the genetic reprogramming of somatic cells through the ectopic expression of a specific combination of transcription factors (Ibrahim et al. 2016). Enchanted by the extraordinary initial work of Takahashi and Yamanaka, many research groups followed their transcription factor-based reprogramming approach and reproduced the results in mice using cells from diverse tissue sources (Yu et al. 2007; Wernig et al. 2007; Maherali et al. 2007; Ahmed et al. 2011a; Buccini et al. 2012) and humans (Lowry et al. 2008; Park et al. 2008). The reprogramming technique provided an unparalleled and distinctive opportunity to researchers in the field of stem cells and regenerative medicine for possible applications, including pediatric applications, to manufacture patient-specific stem cells for human-disease modeling, drug screening and development, and customized cell therapy (Cagavi et al. 2018; Omole and Fakoya 2018; Çetinkaya and Haider 2020).

Since iPSCs appear to end the disputes over the destruction of embryos in hESC research, human-induced pluripotent stem cells (hiPSCs) have been touted by scientists and ethicists alike as ethically and morally uncomplicated alternatives to hESCs and are tipped as surrogate ESCs, and the ethics surrounding hiPSCs have been primarily evaluated in comparison with hESCs. However, even if future investigations demonstrate that hiPSCs fulfill the expectation that they could be possibly viable and superior substitutes for hESCs in disease research, regenerative medicine, and drug discovery, further scrutiny of the reprogramming technology and the resulting ethical concerns might potentially reduce some of the hiPSC-associated ethical advantages over hESCs (Zacharias et al. 2011). In the earliest report on iPSC generation, tumor formation was noticed in more than 20% of the iPSCs due to the reactivation and overexpression of c-Myc oncogene (Okita et al. 2007; Ahmed et al. 2011; Buccini et al. 2012).

There is also the safety risk of insertional mutagenesis from virus-dependent delivery methods, which can lead to tumor formation (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007). Ethical and legal challenges are also associated with the potentiality of using hiPSCs for the development of human-animal chimeras, human reproductive cloning, and the derivation of human gametes (Lo et al. 2010; Ishii et al. 2013; Wu et al. 2016; Zheng 2016; Volarevic et al. 2018; Moradi et al. 2019). Additionally, such concerns as the application of intellectual property rights or hiPSC patents, donor information, and consent pose considerable challenges to the advancement of iPSCs and iPSC-based research

(Lo and Parham 2009; Zarzeczny et al. 2009; King and Perrin 2014; Orzechowski et al. 2020). While many of these ethical challenges are not unique to iPSCs but are also shared by hESCs, the ease of accessibility and the simplicity of procuring starting cell sources for iPSC development, the rapid progress in iPSC research witnessed in the last decade, and the remarkable expectations placed on iPSC technology make it very timely and crucial to consider the ethical and legal issues associated with it. Notably, hiPSCs may provide a renewable source of cells for theranostic applications with moral and ethical advantages over their counterpart pluripotent stem cells. Indeed, hiPSCs have some serious ethical concerns that scientists and bioethicists must recognize. This chapter summarizes some of the primary ethical issues associated with the use of hiPSCs, such as safety, reproduction, patenting, and informed consent/donor's right, which generally remain unfamiliar to a common reader in the field.

Safety

There remains significant uncertainty regarding the properties of hiPSCs, how they are reprogrammed, and their ability to form teratomas. The early iPSC lines were generated by transducing somatic cells using retroviral-vector-carrying gene encoding for various transcription factors (Takahashi and Yamanaka 2006; Takahashi et al. 2007). However, insertional mutagenesis using an integrative gene delivery system is a substantial safety risk of this approach, which may even result in tumorigenicity (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007; Omole and Fakoya 2018). About 20% of the offspring generated in the original report on germline-competent iPSCs subsequently developed tumors, which were attributed to the reactivation of c-Myc transgene (Okita et al. 2007). Such data prompted many research groups to eliminate c-Myc from the classical quartet of transcription factors to enhance their safety profile (Nakagawa et al. 2008; Martinez-Fernandez et al. 2009). These safety risks are unique to iPSCs due to the combined effect of the overexpression of reprogramming factors and the integrative viral-vector-based delivery method used in the protocol for iPSC generation. Furthermore, incorrect or incomplete patterning and genetic instability can increase the risk of tumorigenicity (Yamanaka 2020).

Incorrect or incomplete patterning involves the persistence of undifferentiated and immature cells in the end product of the reprogramming (iPSCs) as well as the differentiated cells derived from hiPSCs. These undifferentiated contaminating cell population has been associated with teratoma formation. The risk of genetic mutations altered biology, and the attainment of tumorigenic potential from incomplete patterning and genetic abnormalities is not unique to iPSCs but relatively common to all cells, which require long-term expansion in vitro (Wang et al. 2013; Izadpanah et al. 2008; Røsland et al. 2009). Genetic alterations like chromosomal aberrations, single nucleotide mutations, and copy number variations are common during reprogramming (Turinetto et al. 2017; Yoshihara et al. 2017a; González and Haider 2021). Chromosomal alterations can either exist in the somatic cells prior to their use

for iPSC generation or originate during the reprogramming process (Yoshihara et al. 2017b; Liu et al. 2020). Indeed, the first hiPSC clinical trial in 2014 was momentarily halted after discovering mutations in the hiPSCs of the second patient, although mutations were absent in the primary somatic cells (Kimbrel and Lanza 2015; Attwood and Edel 2019). Following the transplantation of hiPSCs, the expectation is that the cells should develop normally, maintain average growth, function in the in vivo environment, and adequately replace the injured or lost cells in the diseased patient.

Nevertheless, these cells may proliferate and increase uncontrollably, creating a tumor at the implantation site. This risk of tumorigenicity might trigger extensive safety and ethical concerns about the use of hiPSCs, hence slowing the progress of its application in stem-cell-based clinical therapy. Interestingly, stem cell scientists have made some progress in addressing some of these limitations caused by tumorigenicity. The c-Myc transgene has been shown to be dispensable for reprogramming (Nakagawa et al. 2008). Thomson's group developed their iPSCs using a different set of four reprogramming factors: Oct3/4, Sox2, Nanog, and Lin28 (OSNL), substituting Nanog and Lin28 for c-Myc and Klf4 in Yamanaka's "OSKM" cocktails (Yu et al. 2007). Nonviral delivery methods (plasmid vectors, transposons), non-integrative delivery methods (Sendai virus, lentivirus, and adenovirus), and protocols based on small molecular treatment of somatic cells have been employed to eliminate the limitations caused by insertional mutagenesis (Pasha et al. 2011; Chen et al. 2013; Driscoll et al. 2015; Lee et al. 2020; Kim et al. 2020; Yoshimatsu et al. 2021).

Further intensive studies are fundamental for refining the reprogramming techniques of somatic cells and discovering how to prevent the tumorigenicity of hiPSCs. Another approach in this regard is to develop protocols for the direct reprogramming of somatic cells to the lineage of interest without passing through a pluripotency state (Ahmed et al. 2012). Reliable safety assays should be developed to evaluate the potential of hiPSCs before their application for cell therapy. The development of more effective protocols for iPSC differentiation must first be ensured to generate purified populations of hiPSCs before they are used clinically.

Regarding incorrect patterning, stem cell researchers are developing means to address tumorigenicity to meet the safety standards required for clinical therapy using iPSCs. Some purification methods have been adopted to identify and remove the residual undifferentiated pluripotent stem cells (PSCs). They include techniques such as directed differentiation and positive/negative selection markers using antibody cell sorting systems, such as fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) (Abujarour et al. 2013; Wuputra et al. 2020). The researchers in the clinical study on spinal cord injury are contemplating the use of the suicide gene method as an additional method to prevent tumorigenicity (Kojima et al. 2019). All these methods will assist the investigators in carefully selecting iPSC lines with the highest level of purity that will be safe for the purpose of clinical application.

Regarding genomic alterations, traditional methods like chromosomal karyotyping can detect abnormalities like deletion, duplication, and rearrangement,

and iPSC products with such abnormalities can be discarded. Minimal genetic alterations, like single nucleotide variation (SNV) and copy number variations (CNV), can be detected by next-generation sequencing technology (like whole-genome sequencing) (Yamanaka 2020). However, analyzing such minimal genetic abnormality can be difficult due to the present difficulty experienced; currently, we have to sequence a significant portion of our genome and accurately analyze and interpret the risks from the mutations detected (D'Antonio et al. 2018). The understanding and assessment of the mutational burden of iPSCs are important for their use for therapeutic applications. Indeed, it is challenging to ensure whether a mutation/mutations detected in the iPSC products will significantly increase the risk of tumorigenicity after transplantation (Yamanaka 2020). At present, extensive tests must be carried out on the iPSC products to detect significant mutations, and only stem cells that pass the test should be forwarded for clinical use. Furthermore, after successful transplantation of the iPSCs, patients should be monitored for the possibility of developing a tumor. More clinical research work is needed to accurately predict the tumorigenic possibilities of a detected mutation.

Reproduction

Indeed, one of the most distinct and ethically worrisome potential uses of iPSCs is the production of human embryos through human reproductive cloning. The use of iPSCs for human cloning is illegal and is prohibited worldwide. Generating full-term mice (considered the most stringent criterion of pluripotency) has been fulfilled using iPSCs through tetraploid complementation assays (Kang et al. 2009; Zhao et al. 2009, 2010). This assay involves the injection of iPSCs into the blastocysts of tetraploid mice, embryos that cannot develop into a fetus by themselves. The union results in reconstructed embryos that later develop into fetuses, confirming that iPSCs can form new lives. Sir John Gurdon achieved the first example of cloning using a method where somatic cells were reprogrammed to the embryonic pluripotent states with the same genetic makeup, which is termed somatic cell nuclear transfer (SCNT) (Gurdon 1962). This was followed by Sir Ian Wilmut, who used the same SCNT method to generate the first mammalian – Dolly the sheep – by somatic cloning (Wilmut et al. 1997).

SCNT involves the transfer of somatic nuclei into enucleated oocytes to reconstruct embryos (Matoba and Zhang 2018). Theoretically, this procedure is applicable to humans. Yes, human cloning from hiPSCs is technically possible despite associated safety risks (Wilmut et al. 2015). In both tetraploid complementation and SCNT, normal human oocytes or embryos will be destroyed. In tetraploid complementation, human tetraploid embryos will be generated by the fusion of human diploid embryos. Thus, the normal diploid embryo will be destroyed in the process. The low viability rate during this process will require generating many reconstructed embryos to ensure an increased birth rate of the cloned offspring. Hence, the destruction of many diploid human embryos in the process remains a limitation. Likewise, in SCNT, the enucleated oocytes are also destroyed. This is tantamount to

sacrificing many lives for one life, thus raising ethical concerns that are comparable to that of hESCs. These concerns include controversies about the onset of human personhood and the treatment and harm to embryos, concerns about the safety and health risks of women donating eggs and their potential exploitation for their ova and their informed consent, and concerns about harm to respect for human life, human dignity, and justice toward humankind. In addition, people may also choose to use genetically modified hiPSCs in human cloning to develop offspring with unique characteristics, therefore treating the cloned offspring as a tool for genetic modification or diversity. This type of gene customization of offspring will not show respect for human life. Good surveillance and regulatory processes are essential to monitoring research projects involving SCNT and tetraploid complementation. Regulations must be developed to ban human reproductive cloning explicitly.

Another ethically fraught potential use of iPSCs is the derivation of human gametes (sperm and eggs) and human-animal chimeras. The "first generation" of iPSCs did not contribute to the germline or produce adult chimeras. Yamanaka and others later modified the induction protocols, leading to the generation of iPSCs that were fully reprogrammed and proficient for adult chimera and germline transmission (Okita et al. 2007; Takahashi et al. 2007; Yu et al. 2007). Much progress has since been made in the differentiation of pluripotent stem cells into human sperms and oocytes. Protocols have now been established to successfully differentiate and develop male and female gametes from iPSCs (Panula et al. 2011; Hayashi et al. 2011, 2012; Irie et al. 2015; Sasaki et al. 2015; Yamashiro et al. 2018). It is pertinent to mention that gamete derivation from iPSCs may serve as a powerful research tool to improve our understanding of human development and assisted reproductive techniques for the management of infertility disorders (Fang et al. 2018; Zhang et al. 2020). Nevertheless, the chance that they may be considered for reproductive intents poses ethical concerns about cloning, safety, donors' consent, and the right of the unborn child to know the parents (Advena-Regnery et al. 2018). Other ethical concerns include the potential risk of changing the natural reproduction method, the generation of gametes for same-sex reproduction, and asexual reproduction (Mathews et al. 2009). Furthermore, since the induction of the hiPSCs into gamete cells is not presently a highly efficient process, an attempt to make embryos from such will result in the extensive destruction of many poor-quality embryos, thus raising the same ethical concerns as for hESCs (Mathews et al. 2009).

Chimeras are single organisms containing cells from two or more organisms – that is, it contains two or more sets of DNAs, with the genetic code to make two or more separate organisms. Human-animal chimeras have been used enormously by scientists to improve our understanding of gene function and regulation and disease mechanisms and for testing experimental drugs and gene therapies (Levine and Grabel 2017). They are excellent models of human tissues than nonchimeric animals because they are improved systems for human disease modeling. They provide the opportunity to research human cells and tissues in vivo without the necessity for human experimentation. The technology of interspecies blastocyst complementation has already been used to develop rat organs in mice and vice

versa (Kobayashi et al. 2010; Isotani et al. 2011; Yamaguchi et al. 2017), though human-mouse chimera research is the routine.

Recent advances in genome-editing and stem-cell technology have led to extending this research to larger animals, such as pigs. The combination of gene-editing technology and interspecies blastocyst complementation has made it possible to use hiPSCs to generate individualized human organs, thus raising the opportunity of addressing the dire shortage of organs for transplantation (Wu et al. 2017). However, the growing amounts of human tissues in these chimeras and the potential availabilities of these tissues in morally significant sites, such as the brain, raises strong ethical concerns and questions about the moral status of these animals (Savulescu 2016). How many human cells are considered "too many" in a human-animal chimera's brain? How many human cells are considered "too many" in the human-animal chimera's body altogether? How many would human cells make a mouse brain start thinking human thoughts? What would happen if an animal with human nervous tissues become self-aware and start thinking and feeling like a person? How do we know if we have crossed the commonly accepted dividing line of human decency, dignity, and morality regarding human-animal chimera research? No one knows the answers to these questions, at least not yet.

Nevertheless, these questions reveal the main ethical dilemmas that bioethicists are worried about – that chimeric animals with humanized organs may develop human-like consciousness, which will be ethically unacceptable (Bourret et al. 2016; Kwisda et al. 2020). For further in-depth analysis and detailed arguments and public debates on these concerns, please refer to the works reported by Marino, Knoepfler, Palacios-Gonzalez, DeGrazia, and Greely (Degrazia 2007; Palacios-González 2015; Knoepfler 2016; Marino et al. 2017; Koplin and Wilkinson 2019; Greely and Farahany 2021). Going forward, further debates and research are essential to tackle this major ethical dilemma connected with human-animal chimerism. Therefore, we strongly recommend the practical recommendations for chimeric research contributed by Hyun and colleagues (2007).

Overall, the ethical objections to all the issues raised concerning reproduction include the sanctity of human life, human dignity, safety, manipulation of genetic diversity, violation of the clone's rights, etc. (Pattinson 2007). Despite these ethical objections, the Human Fertilization and Embryology Authorities (HFEA) in 2007 agreed for a cytoplasmic hybrid research program to proceed in the United Kingdom (Editorial (Lancet) 2007; Mayor 2008). Meanwhile, in the United States (September 2015), the National Institutes of Health (NIH) announced the discontinuation of the research funding of iPSC-derived chimeras due to additional controversial ethical issues, which require the attention of enforced policies (NIH, Human Pluripotent Cells into Non-human Vertebrate Animal 2015a, Web, July 1, 2021; NIH, Staying Ahead of the Curve on Chimeras 2015b, Web, July 1, 2021). The authors agree that all aspects of stem cell research should be covered by legislation and strict licensing procedures to curtail the potential for the abuse of this technology. However, we also believe that a flexible, less restrictive regulation that considers the proper justification for embryo research will eventually benefit all.

Patentability

A patent gives an inventor the monopoly right to commercialize an invention for a limited period. Comparable to other property types, a patent makes the inventor the owner of the invention, while the intellectual property right remains valid. This concept of ownership has sparked ethical debate in relation to the patentability of life, centered on the objectification and commercial exploitation of living creatures (Schrecker et al. 1997). Intellectual property rights, when efficiently applied, can present a stumbling block to the progress of iPSC research. There are many approaches used for the generation of iPSCs. If investors hold several patents for these many iPSC generation methods, this can impede the translation of the technology from bench to bedside. Although European patent law (Fig. 1) is set up to protect a person's dignity, the development of iPSCs has opened a worrying loophole (Meskus and de Miguel Beriain 2013). The European Union Court of Justice, on October 18, 2011, delivered a crucial judgment in the aspect of human embryo

Fig. 1 UK and European legal framework for stem cell line patenting

protection in the case C-34/10 *Oliver Brüstle* vs. *Greenpeace eV.* By referring to the meaning of Article 6(2)(c) of Directive 98/44/EC, this case law clarified that those inventions, which involved human embryo destruction at any point, could not be patented (Spranger 2012). However, iPSCs were not in the contemplation of lawmakers when the Biotechnology Directive (Council Directive 98/44/EC and Parliament, from July 6, 1998) was drafted in 1999 (https://eur-lex.europa.eu/legal-contenEN/TXT/?uri=celex%3A31998L0044). Based on the ruling, patents on stem cells generated from excess embryos from IVF or SCNT or through parthenogenesis will be banned. However, since iPSCs were not derived from embryos, the ruling leaves the door open to patents on iPSCs. Subsequently, in the United Kingdom, regulatory guidance has been offered, which opens the door for the patenting of iPSCs, potentially reviving ethical concerns (UKIPO. Inventions involving human embryonic stem cells, 2015, March 25, 2015).

The authors recommend a participatory, inclusive, and transparent process in establishing a workable iPSC patent system that considers the different moral values of all stakeholders in the stem cell field. Creating such a system may not be an easy task, considering the different moral values of all stakeholders. However, if accomplished, this will facilitate the bridging of a moral divide and ensure a consensus that benefits all. More debate and research are essential if we are to close the gap between patents and innovations.

Informed Consent and Donors' Right

Like any other research involving humans, consent is vital for hiPSC research, whether humans participate as research subjects or donors. Usual ethical standards require that participants are fully informed about the specific details of the proposed study, and they are expected to provide voluntary and well-informed consent to participate in the study. Informed consent ensures that the rights, interests, and dignity of patients are protected and respected. Individuals donating somatic cells for iPSC generation should have enough information and answers to address their concerns. The UK Stem Cell Toolkit (USCTK) summarizes iPSC applications concerning legislation (NIH, UK Stem Cell Toolkit 2018, July 1, 2021). These regulations can be used to determine what to include in a consent form. Informed consent should state if the donated cells involved research or clinical applications, genetic modification, animal testing, in vitro or in vivo trials, and whether it will be involved in a therapeutic or diagnostic product with any potential licensing and if there will be risks, complications, and uncertainties. Donors should refuse specific applications, and the right to withdraw one's cell lines should be discussed clearly in the form. If other applications were not mentioned in the initial document, consenting the donor to be recontacted for such an effect could prevent conflict (Zarzeczny et al. 2009; Aalto-Setälä et al. 2009; Orzechowski et al. 2020). Clear explanations and consent will need to be provided as well for patients treated with iPSCs.

Under what circumstances can the participants withdraw from a study? Should a time limit be considered for patient withdrawal? It can get quite complicated when it comes to withdrawal in stem cell research or cell therapy trials. All the steps involved, from obtaining somatic cells from donors to using them to generate iPSCs, are very expensive and time-consuming. So imagine a worse scenario where several donors request a withdrawal after establishing iPSC lines and, at the point, where the iPSCs are to be employed for a clinical study. Such a withdrawal will be very damaging to the research project, and it will be a complete waste of time, money, and other resources (Sugarman 2008). Although the usual standards of research ethics require that participants withdraw from the study at any time, and thus this right must be recognized, there can be "points of no return" that research participants should be informed about (Zarzeczny et al. 2009; Moradi et al. 2019). Points of no return can be when transplanted cells (in cell therapy trials) cannot be removed from the patient's body, thus receiving an irreversible treatment. Even cells donated for research (e.g., to a stem cell bank) may be challenging to withdraw if they have already been used to create a cell line. If there are any such points of no return relevant to given research projects, prospective participants should be informed about them. All this vital information, and the time limit for withdrawal, should be well specified in the consent form (Caulfield et al. 2007). It is indeed a challenge to balance the varied interest linked with iPSC research, considering the prospective benefits of the investigation as well as the interest of the donor. Nevertheless, the apparent policy positions should be adopted and followed through consistently to avoid unnecessary impediments to the research while ensuring the respect and protection of donors' rights.

Closely related to informed consent is the donor's right to control the scope of the research carried out on their cells as well as the scientific and commercial uses of stem cell lines derived from their cells. The stem cell lines will carry the deoxyribonucleic acid (DNA) of the donor, which contains a wealth of information, including the genetic susceptibility of the donor to disease. The disclosure of such information could inappropriately breach the donors' right to their privacy (Sugarman 2008). In the USA, the federal law termed "Genetic Information Non-discrimination Act of 2008" is a typical example of a legislative way of addressing such issues (Taylor 2012). Donors' rights regarding iPSC research may be exercised in various ways. Some donors may not permit their cells to be injected into humans, and they may oppose all animal research or the mixing of human and animal genetic materials. These objections may lead to friction between obtaining the benefit of iPSC research and respecting the donor's autonomy.

One excellent way to address this issue is to preferentially utilize somatic cells only from donors willing to support and allow all forms of basic research into stem cells. However, this strategy has its risks. There is a danger of introducing bias in research if one decides to select only cells from donors who allow all forms of basic research. Additionally, what if those cells do not exhibit the properties needed for the research project? What if the recruitment of this type of subjects who agree to all forms of research takes considerable time and slows down the research project?

Another approach is for researchers to ensure they give precise and thorough explanations about the nature of stem cell research when obtaining informed consent. Although an informed consent procedure that provides complete and relevant information, which enables autonomous decision-making, should be the goal of every recruitment process, this standard is probably not generally lived up to. Providing comprehensive explanations about the nature of stem cell research to the prospective participant can be a difficult task. Information about stem cell research can be quite complicated, and some details may not be understood if one does not provide some background details. In general, whatever approach is considered, the pros and cons should be thoroughly debated before recruiting patients for the study.

Conclusion

The use of stem cells remains a controversial topic despite the advent of hiPSCs. While their generation does not involve the destruction of an embryo, as with ESCs, debates on how they should be used are still relevant (Hug and Hermerén 2011). To address all the issues considered in this chapter and ensure that hiPSCs are not exploited or used unethically, pertinent regulations must be implemented. Perhaps the recent workshop held by the NIH can serve as a model for proactive policy evaluation (NIH VideoCast – Workshop on Animals Containing Human Cells 2015; NOT-OD-15-158: NIH Research Involving Introduction of Human Pluripotent Cells into Non-human Vertebrate Animal Pre-gastrulation Embryos). If stem cell scientists, bioethicists, and policy makers can maintain an open dialogue about the current state of research, then potential ethical issues on the horizon can be tackled in advance. Such an approach would allow hiPSCs for human treatment to be appropriately moderated without blocking vital research progress that will benefit all.

Cross-References

- ▶ Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs) as a Platform for Modeling Arrhythmias
- ▶ Induced Pluripotent Stem Cells

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Response of the Bone Marrow Stem Cells and the Microenvironment to Stress

40

Marrow Adipose Tissue and Leukemia

Duygu Uçkan-Çetinkaya and Bihter Muratoğlu

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D. Uçkan-Çetinkaya (✉)
Faculty of Medicine, Department of Pediatrics, Division of Hematology, Hacettepe University, Ankara, Turkey

Center for Stem Cell Research and Development (PEDI-STEM), Hacettepe University, Ankara, Turkey

Institute of Health Sciences, Department of Stem Cell Sciences, Hacettepe University, Ankara, Turkey
e-mail: duckan@hacettepe.edu.tr

B. Muratoğlu
Center for Stem Cell Research and Development (PEDI-STEM), Hacettepe University, Ankara, Turkey

Institute of Health Sciences, Department of Stem Cell Sciences, Hacettepe University, Ankara, Turkey
e-mail: bihtermuratoglu@hacettepe.edu.tr

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Abstract

In recent studies, the critical roles of the bone marrow microenvironment (BMME) and mesenchymal stem cells (MSCs) in the pathophysiology of leukemia have been addressed. Yet, the mechanisms need to be defined. Leukemia results from a clonal transformation of hematopoietic precursors by the acquisition of chromosomal rearrangements, genetic mutations, and epigenetic modifications. It has been shown that the initiating intrinsic event then leads to alteration of the BMME to support the survival of leukemic blasts. There is limited data for a primary/initiator role for the BMME leading to the development of leukemia. In humans, the development of myelodysplastic syndrome/secondary acute myeloblastic leukemia years after chemotherapy use, besides intrinsic factors, may point to a critical role of the altered/dysregulated ME in the development of leukemia. Similarly, in inherited diseases with DNA repair defects (e.g., Fanconi anemia), long-term xenobiotic exposure and accumulation in the BMME may contribute to the development of leukemia. These factors suggest a previously underestimated but essential role for the BMME in hematological malignancies. Recently, the role of marrow adipose tissue (MAT), by providing free fatty acids through lipolysis, is being addressed as an important factor supporting leukemic blast survival. On the other hand, adipose tissue inflammation is another topic of interest reported to be associated with obesity, insulin resistance, metabolic syndrome, cardiovascular defects, and cancer. In this chapter, the metabolic response of BMME to stress will be discussed with particular attention to MSCs, MAT, and clinical implications in leukemia.

Keywords

Adipocytes · Bone marrow microenvironment (niche) · Leukemia · Marrow adipose tissue · Mesenchymal stem cells · Metabolic · Stress · Xenobiotic

List of Abbreviations

AhR	Aryl hydrocarbon receptor
AML	Acute myeloblastic leukemia
ANGPT	Angiopoietin
ARNT	The AhR nuclear translocator
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATGL	Adipose triglyceride lipase
BM	Bone marrow
BMAL1	Brain and muscle ARNT-like 1
BMME	Bone marrow microenvironment
BRCA	Breast cancer
CAR	cxcl12-abundant reticular
CCL	C-C motif chemokine ligand
CCL2	C-C motif chemokine ligand 2

CCL3	C-C motif chemokine ligand 3
CFU-F	Colony-forming unit-fibroblast
CHOP	C/EBP homologous protein
CLOCK	Circadian locomotor output cycles kaput
cMAT	Constitutive marrow adipose tissue
CRY	Cryptochrome
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
CYP	Cytochrome P450
DAMP	Danger-associated molecular patterns
DDR	DNA damage response
ECM	Extracellular membrane
ECV	Extracellular vesicle
eIF2-α	Eukaryotic translation initiation factor-2α
EPCs	Endothelial progenitor cells
ER	Endoplasmic reticulum
FA	Fanconi anemia
FABP4	Fatty acid-binding protein 4
FAO	Fatty acid oxidation
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FFA	Free fatty acids
FLT3 TKIs	FMS-like tyrosine kinase 3 inhibitors
FOXC2	Forkhead box protein C2
GCSF	Granulocyte colony stimulating factor
GMPs	Granulocyte-macrophage progenitors
GSTs	Glutathione S-transferases
HIF1	Hypoxia-inducible factor
HIF1-alpha	Hypoxia-inducible factor 1 alpha
HIF2-alpha	Hypoxia-inducible factor 2 alpha
HSCs	Hematopoietic stem cells
HSL	Hormone-sensitive lipase
HSP	Heat shock protein
ICL	Interstrand crosslink
Id1	Inhibitor of DNA binding 1
IGF1	Insulin-like growth factor 1
IL-1 beta	Interleukin 1 beta
IL10	Interleukin 10
IL6	Interleukin 6
IL8	Interleukin 8
IR	Ionizing radiation
IRE1-alpha	Inositol-requiring enzyme 1 alpha
ITD	Internal tandem duplication
LepR	Leptin receptor
LT-HSCs	Long-term hematopoietic stem cells

MDR	Multidrug resistance
MDS	Myelodysplastic syndrome
MEIS1	Myeloid ecotropic viral integration site 1
MGL	Monoacylglycerol lipase
MK	Megakaryocyte
MMP	Matrix metalloproteinases
MPL	Myeloproliferative leukemia protein
MSCs	Mesenchymal stem/stromal cells
NADH	Nicotinamide adenine dinucleotide
NE	Norepinephrine
NES	Nestin
NF-kB	Nuclear factor kappa B
O_2	Oxygen
PAH	Polycyclic aromatic hydrocarbons
PAMP	Pathogen-associated molecular patterns
PER	Period
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC	Peroxisome proliferator-activated receptor gamma coactivator
POPs	Persistent organic pollutants
PPAR	Peroxisome proliferator-activated receptor
PRDM16	PR domain containing protein 16
PTPN	Protein tyrosine phosphatase nonreceptor type
rMAT	Regulated marrow adipose tissue
ROS	Reactive oxygen species
sAML	Secondary AML
SCF	Stem cell factor
SCF	Stem cell factor
SDS	Shwachman-Diamond syndrome
SMAD	Small MAD family
SNS	Sympathetic nervous system
t-AML	Therapy-related AML
t-MNS	Therapy-related myeloid neoplasms
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TGF-β	Transforming growth factor
Tie2	TIE receptor tyrosine kinase
TLR	Toll-like receptor
TNF-alpha	Tumor necrosis factor alpha
TPO	Thrombopoietin
UCP-1	Uncoupling protein 1
UPR	Unfolded protein response
UPR	Unfolded protein response
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule

VCAM1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VLA4	Very late antigen 4
XBP1	X-box binding protein 1
XREs	Xenobiotic response elements

Introduction

Being the main reservoir of stem cells, the bone marrow (BM) is a critical tissue in maintaining homeostasis and coordinating stress response in the organism. In addition to its essential role in the production of hematopoietic stem cells (HSCs) and progenitors, it is also a major depot for mesenchymal stem/stromal cells (MSCs) and endothelial progenitor cells (EPCs), making this tissue an essential player in regenerative medicine. Therefore, BM is a critical tissue in determining the organism's response to stress.

Cells are exposed to several types of stress, including oxidative, inflammatory, genotoxic, hypoxic, replicative, or nutrient stress. Uncovering the stress response of cells and tissues is critical to understand the mechanisms of diseases. The autonomic nervous system and hypothalamic-pituitary-adrenal axis, through sympathetic, parasympathetic mediators, and cortisol release, coordinate the stress response, respectively. The released hormones and mediators act on several organs and tissues (Herman et al. 2003; Ulrich-Lai et al. 2009). Bone marrow has neural innervation by the sympathetic nervous system (SNS) under circadian control, which is critical for cell trafficking, both in homeostasis and in pathological states (Lucas et al. 2008; Méndez-Ferrer et al. 2008). The changing demands of hematopoiesis upon exposure to stressors are met by the spectacular ability of the BM microenvironment in coordinating hematological, immunological functions and metabolism (Zhao and Baltimore 2015). Stress may be overcome through compensatory mechanisms. However, upon chronic exposure to stress, the response mechanisms may fail to deal with the stressful condition and lead to chronic inflammation in the BM that may contribute to hematological abnormalities, such as leukemia.

Among several components of the BM microenvironment, adipocytes have been attracting a lot of attention lately in malignant transformation of hematopoiesis and metastasis of solid tumors (Cawthorn et al. 2016; Shafat et al. 2017). Adipocytes adjacent to tumor cells are involved in metabolic cross-talk, providing adipokines and lipids. Recent evidence suggests that adipose tissue plays a role in cancer aggressiveness by promoting tumor growth, metastasis, increasing cellular lipid metabolism, and chemoresistance (Lengyel et al. 2018).

In this chapter, we summarize the dynamic interactions and alterations in the BM microenvironment in both steady state and upon exposure to stressors and discuss the role of stromal factors in developing myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML).

Bone Marrow Microenvironment

Components of the Bone Marrow Microenvironment

The BM microenvironment is a specialized environment since it provides a niche for stem/progenitor cells, including HSCs, MSCs, and EPCs. It is an enriched and dynamic milieu responding to physiological or stress signals coordinated according to the need of the relevant tissue. Therefore, in addition to its primary function in hematopoiesis, it is essential in maintaining homeostasis, making it a significant player in regenerative medicine. The BM microenvironment is composed of cells of hematopoietic and non-hematopoietic origin (MSCs, pericytes, adipocytes, osteoblasts, macrophages, endothelial cells, osteoclasts, nerve cells, chondrocytes, fibroblasts), a specialized extracellular matrix (ECM) rich in proteins (fibronectin, collagens I-XI, laminin, tenascin, thrombospondin, elastin), proteoglycans (hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate), heparin, glycoproteins, integrins, adhesion molecules, and a soluble environment composed of cytokines, chemokines, growth factors, hormones, metabolites, and extracellular vesicles (ECV), all of which play a critical role in determining stem cell functions such as dormancy, proliferation, adhesion, differentiation, autophagy, apoptosis, and migration (Klamer and Voermans 2014; Crane et al. 2017). Therefore, the marrow is a tissue where active cell trafficking occurs not only under stress but also in a steady state to keep up with the physiological cell turnover. This dynamic microenvironment is regulated through the circadian clock, influenced by extrinsic and intrinsic factors influencing cell to cell, cell to ECM, and cell to soluble microenvironment interactions in the niche (Haus et al. 1983; Méndez-Ferrer et al. 2008).

With an understanding of the critical roles of the BM microenvironment in stem cell biology, many studies have been performed in the last two decades to define the physical, biochemical, and biological characteristics of the BM microenvironment. However, most studies about marrow stem cell niches were hypothetical until the recent technological developments in multi-omics and single-cell study methods, imaging techniques, which led to a better understanding of hematopoiesis and its microenvironment (Liu et al. 2020a). Progress in discovering genetic tools for functionally identifying niche cells in vivo (mice and zebrafish experiments) and new imaging techniques led to better identification of stem cell niche factors (Crane et al. 2017; Wolock et al. 2019).

The recent discovery of the continuous differentiation model of hematopoiesis has suggested that HSCs may bypass discrete hierarchy and gradually acquire lineage biases along with multiple directions (Laurenti et al. 2019). These new developments in hematopoiesis research necessitate a new understanding of the stromal compartment as well. An important study in this area is the extensive mapping of the differentiation paths of non-hematopoietic cells and delineating the BM stroma's differentiation hierarchy (Wolock et al. 2019). Another recent study used combined single-cell and spatially resolved transcriptomics techniques for mapping distinct BM niches by determining their molecular, cellular, and spatial

composition. They could profile all major BM-resident cell types, determine their localization, and clarify sources of pro-hematopoietic factors (Baccin et al. 2020).

These studies confirmed the heterogeneity of stromal niche populations and their close functional interaction with the differentiation stage of the nearby stem/progenitor cells emphasizing the critical roles of spatial positioning and the dynamic interactions in the BM microenvironment.

Different Stem Cell Niches in the BM Microenvironment and Their Spatial Position

The spatial position of different specialized niches in the BM with diverse functions has been a well-adopted opinion since the earlier studies (Wilson and Trumpp 2006). However, unlike the intestinal and skin stem cell niches (Clevers 2013; Hsu et al. 2014), it is challenging to identify BM niches due to their semi-fluidic soft connective tissue structure inside the bone cavity. The HSCs are located near the endosteal lining of the BM cavities or are in close contact with the endothelium of the sinusoids. The hypoxic and calcium-rich endosteal/osteoblastic niche creates a protective and glycolytic microenvironment for the long-term HSCs (LT-HSCs), keeping them in a quiescent state with low metabolic activity and away from toxic insults (Wilson and Trumpp 2006). However, it was shown that the perisinusoidal region also hosts HSCs, in fact, more than the endosteal region. It is known that the marrow is a highly vascularized tissue, and the entire inter-bone space is filled with BM that is fully vascularized, leaving no room for vessel-distant-niches (Stegner et al. 2017). Thus, endothelial cells are present both in the perisinusoidal region (venous type) and also nearby of the endosteum (arteriolar type) (Klamer and Voermans 2014). Therefore, the term "central niche" rather than "vascular niche" is preferred when referring to the perisinusoidal region (Kiel et al. 2005; Lo Celso et al. 2009; Ding et al. 2012; Kunisaki et al. 2013; Acar et al. 2015). Transition zone vessels, connecting arterioles with sinusoids, are also described in the BM microenvironment, located close to the bone surface (Méndez-Ferrer et al. 2020). It has been shown that the spatial position of cells in the marrow is closely associated with the stage of differentiation of cells, earlier stage HSCs and B lymphocytic progenitors residing preferentially in the endosteal niche (Zhu et al. 2007; Wu et al. 2009; Ding and Morrison 2013; Gazit et al. 2014; Morrison and Scadden 2014). Two stromal cell populations representing endosteal and sub-endosteal niches of mice play active and complementary roles on HSCs' recruitment from a quiescent niche into a proliferative niche and/or its engagement into the differentiation cascade (Balduino et al. 2012). In the central/perisinusoidal niche, the metabolic milieu with increased reactive oxygen species (ROS) resulting from mitochondrial oxidative phosphorylation plays a role in directing HSCs towards mature hematopoietic cell lineages. It is stated that the leaky sinusoidal blood vessels with fenestrated basal lamina provide a site of blood cell migration between the BM and the circulation upon exposure of HSCs to blood plasma components and increases in ROS levels (Crane et al. 2017).

Although HSCs' quiescence is critical in maintaining the stem cell pool, recent studies have also shown the critical role of long-lived progenitors in maintaining steady-state hematopoiesis (Sun et al. 2014). Thus, the spatial position of progenitors is suggested to be involved in the regulation of proliferation and differentiation during both steady-state and under hematopoietic stress (Hérault et al. 2017a). The frequent turnover of mature neutrophils and macrophages necessitates a unique localization of granulocyte-macrophage progenitors (GMPs) to regulate their proliferation, differentiation, and responding to negative feedback in the niche. Individual GMPs are scattered throughout the BM in a steady state, whereas during regeneration, clusters are formed from expanding GMP patches, locally differentiating into granulocytes. Besides the granulocytic lineage, a revised model of megakaryopoiesis based on spatial location has been described in which a vessel-biased megakaryocyte pool rather than the migration mechanism from a distant endosteal niche is emphasized (Larson and Watson 2006; Stegner et al. 2017). These findings demonstrate the importance of spatial position in the stress response of the BM microenvironment.

MSCs, being the source of the connective tissue cells, are critical microenvironment factors in coordinating the highly organized BM microenvironment network for healthy hematopoiesis. There is significant heterogeneity among MSCs, both phenotypically and functionally. The standard stromal markers, including CD105, CD29, CD44, CD90, CD73, do not discriminate against different subsets of MSCs. The recent International Society for Cell Therapy (ISCT) standardized norms for characterization of MSCs has been instrumental to remove the inconsistencies regarding the nomenclature as well as biological characteristics of MSCs (Haider 2018). According to ISCT recommendation, MSCs show expression of CD73, CD90, CD105 and lack CD34, CD45, HLA-DR, CD14 or CD11b, CD79a or CD19 membrane surface molecules. It was shown that the perisinusoidal niche is supported by leptin receptor-positive (LepR+) stromal cells with active roles in HSC homing and mobilization (Ding et al. 2012; Ding and Morrison 2013), whereas nestin-positive (NES+) cells in the periarteriolar niche promote quiescence (Kunisaki et al. 2013). Nestin is a typical protein of neural cells (ZIaee et al. 2017). When present on MSCs shows higher colony-forming fibroblast (CFU-F) activity, self-renewal, and multilineage differentiation ability (Méndez-Ferrer et al. 2010b; Zhou et al. 2014). A significant overlap between LepR+ cells and NES+ cells and with those with high levels of CXCL12 (CXCL12-abundant reticular/CAR cells (Sugiyama et al. 2006) have been described. LepR+/CAR cells, although they represent a very low population in the BM, they have long processes that are present throughout the bone marrow and become closely associated with HSCs. Both adipocytes and osteoblasts are cell types differentiated from their precursors, MSCs. A recent study reported two types of cells based on the preferential expression of LepR (Adipo-CAR) and osterix (Osteo-CAR). Spatial analysis revealed localization of Adipo-CAR cells to sinusoids and Osteo-CARs to arterioles in the BM (Baccin et al. 2020). The pericytes in the arteriolar endothelium in the endosteal region were reported to be more quiescent similar to their HSC neighbors, were LepR- and innervated by sympathetic nerves. In contrast, sinusoidal pericytes were positive for the perivascular marker LepR and hosted cycling HSCs (Klamer and Voermans 2014).

Despite the sophisticated experimental studies providing evidence about the role of differential localization and different functions of BM stem cell niches, there is a lack of confirmatory studies in humans. There is a need for clinical translation of the significantly accumulated basic knowledge and experimental works to humans. Metabolic profiling is a valuable tool to determine the state of cells in different microenvironment conditions. Studies have shown that cells with a low mitochondrial potential are highly enriched for HSCs and progenitors in both mice and humans. The LT-HSCs utilize glycolysis with low oxygen consumption rates, have low mitochondrial potential, and have better repopulation ability. These studies have pointed out the hypoxic metabolism, hypoxia-inducible factor (HIF1α), MEIS 1, and the unique metabolic profile of the hematopoietic niche (Simsek et al. 2010; Kocabas et al. 2015). To investigate the spatial position of human hematopoietic niches, we analyzed BM samples derived by superficial and deep aspiration from healthy human BM transplant donors by metabolomics and transcriptomics analyses and determined region-specific metabolic profiles. Significant differences in energy metabolism were detected between two anatomically different regions of the marrow. As expected, the superficial/cortical region of the marrow was representative of the endosteal niche and was enriched for the pentose phosphate pathway. In contrast, the citrate cycle pathway was enriched in the deeper aspiration region, likely, the central marrow region. The culture-expanded MSCs from the superficial and deeper marrow aspiration regions showed differences in gene expression profiles (Ayhan et al. 2021).

Dynamic Interactions in the BM Microenvironment

Increasing knowledge of the dynamic interactions and the metabolic state of the BM microenvironment is essential to understand stress response and disease pathophysiology. The BM is a site of high cell turnover and heavy cell/stem cell trafficking. Following BM transplantation, donor HSCs home to the hematopoietic niche in the marrow and, through adhesive interactions with the cells and the ECM, reside in the endosteal, then in perisinusoidal niches to provide lifelong hematopoiesis of donor type. Homing and retention in the hematopoietic niches are mediated through receptor-ligand interactions between cells and the ECM involving chemokines and adhesion molecules (Heazlewood et al. 2014; Sánchez-Aguilera and Méndez-Ferrer 2017; Wei and Frenette 2018; Pinho and Frenette 2019). Stem cell niches in the BM microenvironment interact with each other and metabolic factors are critical in determining the fate of cell populations in the marrow. Hypoxic state/HIF1α is an important determinant in spatial positioning of LT-HSCs in the quiescent niche in which the metabolic milieu is compatible with a glycolytic environment with low NADH, low mitochondrial activity, and active pentose phosphate pathways. On the other hand, mitochondrial plasticity is also a crucial factor in cell fate determination. Migration of HSCs towards ROS high central/perisinusoidal region favors differentiation, proliferation, and mobilization of HSCs to peripheral blood (Bahat and Gross 2019).

Many cell types are involved in the retention or mobilization of stem cells. Osteoblasts, macrophages, NES+ MSCs provide HSCs retention and maintenance signals, whereas activation of perisinusoidal LepR+ MSCs, osteoclasts, endothelial cells are needed for mobilization. Interactions between cells and ECM components determine the adherence characteristics of HSCs to their niche. CXCL12/CXCR4, VCAM1/VLA4, Angpt1/Tie2, SCF/C-kit, TPO/MPL signaling, matrix components consisting of adhesion molecules, integrins, N-cadherins, osteopontin, calcium ions, hypoxic environment, and TGF-β signaling are among the essential mechanisms providing adherence and maintenance in the quiescent hematopoietic niche (Lapidot and Petit 2002; Arai et al. 2004; Lapidot et al. 2005; Dar et al. 2005; Ramirez et al. 2009; Bonig et al. 2009; Li 2011).

Under steady-state conditions, the great majority of HSCs and progenitors reside in the BM; only a small number rhythmically leave the BM. Autonomic signals from the sympathetic nervous system (SNS) in response to "in need" signals regulate the circadian rhythm of HSC and progenitor mobilization by affecting cytokine, chemokine, cell, and ECM interactions. The HSCs neighboring the arteriolar blood vessels are surrounded by the SNS fibers. HSCs' maintenance/retention factors such as SCF and CXCL12 are expressed by NES+/NG2+ (pericyte-specific marker) perivascular MSCs. The non-myelinating Schwann cells also maintain HSC quiescence by activating the TGF-β)/SMAD signaling. Following circadian secretion of norepinephrine (NE) by the SNS, CXCL2 levels oscillate within the BM microenvironment, orchestrating steady-state egress. When activated, HSCs relocate near the NES low LepR+ MSCs in the perisinusoidal area and are rhythmically released to peripheral blood (Méndez-Ferrer et al. 2010a).

Physiological or stress signals, including cortisol release, toxic insults, chemotherapy, radiation, hypoxia, infection-associated danger signals, and a variety of systemic stressors, metabolites are involved in mobilization. This process is carried out by mechanisms that interfere with retention and activate mobilizing mechanisms resulting in the migration of HSCs towards the perisinusoidal region. Research on stem cell mobilizing strategies has broad implications in clinics, not only in hematological conditions but also in regenerative applications (Wang et al. 2006a, b; Elmadbouh et al. 2007; Haider et al. 2008; Tano et al. 2011; Jiang et al. 2012). Granulocyte colony-stimulating factor (GCSF) is the best known and widely used HSCs mobilizing agent used in HSCs transplantation, also in oncology clinics to hasten recovery from neutropenia. It is endogenously secreted from the BM microenvironment in response to physiological and stress signals. GCSF induces significant changes in the BM; it directly or indirectly interferes with nearly all components of the hematopoietic niche, including the cellular members, ECM, and signaling molecules inducing significant physical and metabolic changes (Greenbaum and Link 2011). The first step in the mobilization process is detachment from the BM niches by interfering with the anchoring interactions mediated by CXCL12, VCAM-1, and their receptors. Interaction of GCSF with its receptor on the SNS leads to: suppression of the niche support function of macrophages and osteolineage cells (Katayama et al. 2006; Chow et al. 2011), inhibition of stromal cell synthesis of retention factors such as CXCL12 (Petit et al. 2002), and

interruption of CXCL12/CXCR4 signaling leading to increased proliferation and mobilization to blood associated with reduced BM but increased blood CXCL12 levels and upregulated CXCR4 expression. GCSF induced neutrophil activation, degranulation, and release of granulocyte proteases, such as neutrophil elastase and cathepsin, play a role in the proteolytic inactivation of these factors and creation of the chemokine gradient for egress (Ratajczak 2015; Hatfield et al. 2017; Itkin et al. 2017; Wei and Frenette 2018).

Proteolytic activation of several factors in BM microenvironment is necessary during HSPCs mobilization. Significant changes occur in all ECM components for mobilization, heparan sulfate proteoglycans being a major one. Heparan sulfate proteoglycans are key regulators of the hematopoietic niche, and their functions are dynamically modified depending on the stage of cell maturity and according to physiological or stress signals. It was shown that heparan sulfate chain structures are continuously remodeled in a spatiotemporal fashion while the stem and progenitor cells progress through various differentiation pathways (Papy-Garcia and Albanese 2017). On the other hand, activation of matrix metalloproteinases (MMP-9, MMP-1) also leads to significant alterations in the ECM. Mobilization from the marrow induces substantial changes in the coagulation and the complement systems as well. Plasminogen plays an essential role particularly in the GCSF-induced mobilization by binding to the BM ECM and, after conversion into plasmin, by degrading various proteins including fibrin, laminin, and activating other proteases, such as MMP-3, MMP-9, MMP-12, and MMP-13, and degrading other matrix components, such as collagen. Similarly, the urokinase plasminogen activator, part of the plasminogen activating system, is also involved in cell traffic (Gong and Hoover-Plow 2012). Moreover, activation of the complement cascade proteins C3 and C5 leads to establishing a highly proteolytic microenvironment that degrades CXCL12. Activation of osteoclasts and secretion of cathepsin K and CD26 (dipeptidyl-peptidase 4) are also involved in the cleavage of CXCL12 and mobilization (Lapidot and Petit 2002; Itkin et al. 2017).

Another important function of CD26 in mobilization is its effect on vasculature. The BM endothelial cells are essential players in the regulation of HSC trafficking. CD26 is expressed by endothelial cells and is involved in maintaining vascular barrier function during stress-induced conditions (Itkin et al. 2017). Recently, the critical role of signaling through neuropeptide Y on HSC trafficking has been shown through its effects on BM vasculature, raising the importance of targeting vasculature as a mobilizing strategy. The expression of CD26 by endothelial cells activates NPY-mediated signaling by increasing the bioavailability of the truncated form of NPY. Neuropeptide Y is released from SNS nerves, enhances the activity of MMP-9, and affects HSC function by modulating MSCs, osteoblasts, and macrophages (Singh et al. 2017). Neuropeptide Y-mediated direct regulation of HSCs' quiescence was also reported by the authors (Ulum et al. 2020).

Other groups of molecules involved in the mobilization process are the angiopoietins (Ang) and angiopoietin-like proteins, with important roles in vascular development, angiogenesis, which also affect the BM niche functions (Arai et al. 2004). Angiopoietins are regulators of endothelial cell interactions with supporting perivascular cells.

Angiopoietin-1 and 2 are ligands of endothelial-specific tyrosine kinase receptor, Tie2. Ang1 and Tie2 interaction is important in quiescent niche function and vascular stabilization. Apart from being an endothelial survival factor, Ang1 protects HSCs from cellular stress, thus contributing to the maintenance of the cells in a quiescent state in the BM niche. On the other hand, Ang2 acts on Tie2-expressing resting endothelial cells as an antagonistic ligand to negatively interfere with the vessel stabilizing effects of constitutive Ang1/Tie2 signaling promotes apoptosis of endothelial cells (Lei et al. 2007a). It was initially identified as a disruptive agent of the vasculature. It has been shown that Ang2 plays a complex role in angiogenesis, depending on the physiological and biochemical environment, acting either as an agonist or antagonist of Tie2 induced angiogenesis (Lei et al. 2007b). Co-overexpression of Akt and Ang-1 significantly contributes stem cell proliferation via miR-143 which critically regulates Erk5/CyclinD1 signaling (Lai et al. 2012a, b) and also causes stabilization of HIF1-alpha and supports endothelial commitment of BM stem cells (Lai et al. 2012b). Ang2 is critical for cytokine-induced vascular leakage and controls the vascular response to inflammation associated with cytokine-induced intracellular calcium influx. With these properties, Ang2 has a role in stress-induced mobilization (Benest et al. 2013). Ang-like 4 (Agptl4) is another factor with critical roles in BM vasculature. Recently, it was shown that Angptl4 inhibition led to increased BM vascular permeability and increased trafficking of HSCs and progenitors into circulation in BM homeostasis and as a stress response (Suzuki et al. 2021).

Lipid metabolism also plays a vital role in mobilization. Sphingosine-1 phosphate and ceramide-1 phosphate, the bioactive phosphorylated lipids, are potent chemoattractants involved in HSC, progenitor, and malignant cell trafficking (Shirvaikar et al. 2010; Kim et al. 2010; Ratajczak et al. 2014; Ratajczak 2015; Albakri et al. 2020). Recent studies demonstrated the critical role of lipolysis and the lipolytic enzyme hematopoietic-specific phospholipase C-β2 (PLC-β2) in HSC egress (Adamiak et al. 2016). It was shown to be upregulated in the BM microenvironment during the mobilization process and lead to impairment of membrane lipid raft formation, which is required for the optimal BM retention function of glycolipid glycosylphosphatidylinositol-anchor-associated proteins, CXCR4 and VLA-4. It was also shown that the membrane type 1-MMP upregulation in hematopoietic cells and its enhanced incorporation into membrane lipid rafts contribute to proMMP-2 activation, further facilitates HSCs mobilization upon exposure to GCSF (Shirvaikar et al. 2010). The effect of lipid metabolism in BM trafficking was also demonstrated in the recent study (Suzuki et al. 2021), which reported the important role of dietary fat intake on signaling in BM granulocytes. The BM fat ligand for PPARδ was suggested as a negative regulator of mobilization in fed mice. On the other hand, mice on a fat-free diet showed increased mobilization with PPARδ agonist. The authors point out the clinical relevance of this mechanism in poor mobilizers (Suzuki et al. 2021).

Stem cell trafficking has significant clinical implications. Therefore enlightening the mechanisms and revealing therapeutic targets is an interesting subject. The use of antagonists of chemokine receptors, cytokines, chemokines, bioactive lipids, bacterial toxins, proteases, inhibitors of adhesive cell interactions,

polymeric sugar molecules, and modification of their biological effects are strategies towards optimization of stem cell mobilization in transplantation in preventing cancer metastasis and for organ regeneration (Albakri et al. 2020). Studies on humans consist of investigations performed on GCSF-exposed human blood or BM samples of HSCs transplant donors. Significant alterations in the blood metabolite profile are described in apheresis donors exposed to GCSF consisting of significantly increased levels of several medium and long-chain fatty acids, and polyunsaturated fatty acids and reduced levels of other lipid metabolites, such as phospholipids, lysolipids, and sphingolipids (Hatfield et al. 2017). On the other hand, studies performed by us on BM samples of healthy donors after GCSF exposure revealed alterations in the levels of growth factors, cytokines, and immunomodulatory factors, and upregulation of both hematopoietic colonies and the colony-forming ability of MSCs (fibroblast colony-forming units/CFU-Fs), indicating the regenerative potential of GCSF (Ok Bozkaya et al. 2015; Aerts-Kaya et al. 2021). The role of GCSF in non-hematopoietic tissue/organ regeneration has gained attention in several clinical disciplines. The multifaceted effects of GCSF, particularly on endothelial cells and angiogenesis, are suggestive of its beneficial roles in injury states. Starting in the 2000s and in recent years, GCSF has been used by some centers for organ injury, such as after neurological insults (Lee et al. 2005; Liu et al. 2020b). Favorable results reported were attributed to activation of several mechanisms, including enhanced angiogenesis associated with increased endothelial proliferation, increased endothelial NO synthase, and Ang-2 expression. In addition, the anti-inflammatory and anti-apoptotic effects of GCSF were also identified as contributing factors to its neuroprotective properties.

Bone Marrow and Stress Response

Studies regarding the continuous, dynamic, and organized interactions in the BM niche indicate the critical role of the microenvironment in maintaining homeostasis. Therefore, enlightening the niche regulatory mechanisms under stress is important to delineate the highly active part of the microenvironment in the pathophysiology of cancer and other hematological disturbances.

Cell and Microenvironment Response to Stressors

Cells are continuously exposed to internal, external, physiological stimuli or stressors upon which various stress responses are activated to recover cell function and maintain homeostasis within the tissue and the organism. Depending on the intensity and duration of the stimuli, different signals that induce microenvironment and/or systemic responses are generated usually associated with paracrine and endocrine signals. Upon exposure to low doses of stressors, either the former state of the cells is preserved or shows an altered profile and adapt to the new

environment. In contrast, the repair process may be ineffective in prolonged and/or severe stress leading to cellular senescence or death. The microenvironment of cells is the determinant of the fate of the stressed condition. Persistent cell stress enhances susceptibility to cancer and aging-associated diseases and is usually associated with chronic inflammation.

Cells may be exposed to different types of stressors such as genotoxic stress/DNA damage, hypoxia, oxidative stress, nutrient/metabolic stress, and xenobiotics (Ivanusic 2017). Genotoxic stress is caused by ionizing and ultraviolet radiation, exposure to chemotherapy, other toxic agents, and ROS. DNA damage requires complicated molecular mechanisms to maintain genomic stability through DNA repair. Activation of the DNA damage response (DDR) signaling pathway results in either cell cycle arrest and DNA repair or apoptosis (Zambetti et al. 2016; Davalli et al. 2018; Huang and Zhou 2019). Oxidative stress derives from the excess production and accumulation of ROS or a defect in the antioxidant protective mechanism. While low doses of ROS stimulate cell proliferation, higher doses can result in cell cycle arrest, senescence, or cell death (Finkel 2003). Hypoxic stress leads to cell cycle arrest to minimize energy consumption to preserve oxygen for cell metabolism. The cellular response to hypoxia is mediated by both HIF-dependent and HIF-independent pathways, i.e., mTOR signaling. Hypoxia, respiratory poisons, and xenobiotics are stressors that cause mitochondrial stress (Liu et al. 2006; Zhang and Zhang 2018). The availability of nutrients is crucial for metabolic homeostasis and the proper function of the cell. Nutrient stress causes metabolite fluctuations, which are recognized by the lipid membranes of organelles. One of the most essential nutrients is glucose, and both deprivation and excess of glucose can cause cellular stress. Nutrient deprivation activates autophagy in most cells, enabling them to catabolize their components for survival (Sekine et al. 2021). Heat shock causes protein denaturation, unfolding, and aggregation. The molecular chaperones, heat shock proteins (HSPs) are responsible for protein folding, unfolding, and/or refolding in either standard or stressed conditions (Vabulas et al. 2010). Similarly, chemical toxins also cause protein denaturation and activate the unfolded protein response (UPR) in the endoplasmic reticulum and mitochondria. On the other hand, infectious agents can induce several stress responses by activating pattern recognition receptors (Muralidharan and Mandrekar 2013).

The microenvironment plays a critical role in activating and performing cell-intrinsic or extrinsic stress response mechanisms (Galluzzi et al. 2018). Stressed cells may show altered morphology, phenotype, molecular, metabolic profile, and secretory properties, including the release of ECVs (exosomes) with a variable composition of protein cargo (Abramowicz et al. 2019; Haider and Aramini 2020). Suppression of proliferation and elimination of terminally damaged cells are protective mechanisms against inflammation and cancer. The stress response mechanisms, including the DDR, UPR, mitochondrial stress signaling, may result in temporary adaptation, induce autophagy, or trigger cell death. Autophagy is a significant stress response mechanism important in adaptation to a stressed situation. Potentially harmful or disposable cytoplasmic contents are degraded. Physiological autophagy levels are required for maintaining stable cell homeostasis under stress conditions,

while exacerbated autophagy induces uncontrolled cell death. Autophagy in one cell releases signals that affect other cells locally and systemically. Thus, autophagy affects the local microenvironment, may elicit a systemic metabolic response, and may contribute to the pathophysiology of diseases, e.g., by providing metabolites such as lactate, ketone bodies, alanine to cancer cells as microenvironment support (Capparelli et al. 2012; Sousa et al. 2016). Hence, depending on the intensity of the aggressive stimuli, autophagy can be beneficial or harmful, mediate anti-inflammatory or pro-inflammatory effects, and maybe playing either a cytoprotective role or mediates regulated cell death. Stress-induced regulated cell death, as opposed to programmed cell death, may lead to a release of cellular contents and may trigger pro-inflammatory signals. However, the local microenvironmental factors such as the efficiency of the phagocytic system may affect the outcome of the stress response. Cellular senescence is another stress response of cells wherein DNA damage is irreparable but insufficient to drive regulated cell death, oncogene signaling, or other threats to homeostasis and is associated with permanent growth arrest. However, some metabolic activities of senescent cells, secretion of ROS, and pro-inflammatory cytokines are preserved.

Hematopoietic Stress Response

Constant blood cell production and the continuous cell traffic in the BM render the hematopoietic system highly sensitive to toxic agents. Under homeostasis, most of HSCs are quiescent and rarely enter the cell cycle to self-renew or differentiate. There is a fine-tuning balance between the quiescent, proliferating, differentiating, and senescent cells. In response to external stimuli, such as infection, blood loss, or toxic insults, HSCs can leave a quiescent state and become proliferative. Recent evidence suggests that acute and chronic stress impact the number and function of HSCs, including their ability to repopulate and produce mature cells. The BM microenvironment has a significant role in determining the response and maintaining a steady-state and life-long hematopoiesis (Walter et al. 2015; Pratibh et al. 2020).

HSCs and BM microenvironment may be exposed to different types of stress, including oxidative, hypoxic stress, inflammation, blood loss, ionizing radiation, cytotoxic chemotherapy, all of which lead to BM injury, disrupt homeostasis, and cause significant alterations in cell composition (Mendelson and Frenette 2015; Pratibh et al. 2020) (Fig. 1). The hematopoietic system can quickly respond to infection and inflammation by increasing myeloid and immune cell production. This is a well-known response presenting as a left shift of myeloid elements in the peripheral blood of patients with sepsis and bacteremia. A similar proliferative response in relevant components of the hematopoietic lineages are observed in other pathological conditions, such as increased megakaryocyte production in patients with immune thrombocytopenia as a BM response to platelet consumption platelet, alternatively, accelerated production of erythroid precursors under conditions of immune destruction or blood loss. These clinical manifestations represent the rapid-acting compensatory function of the BM microenvironment to preserve

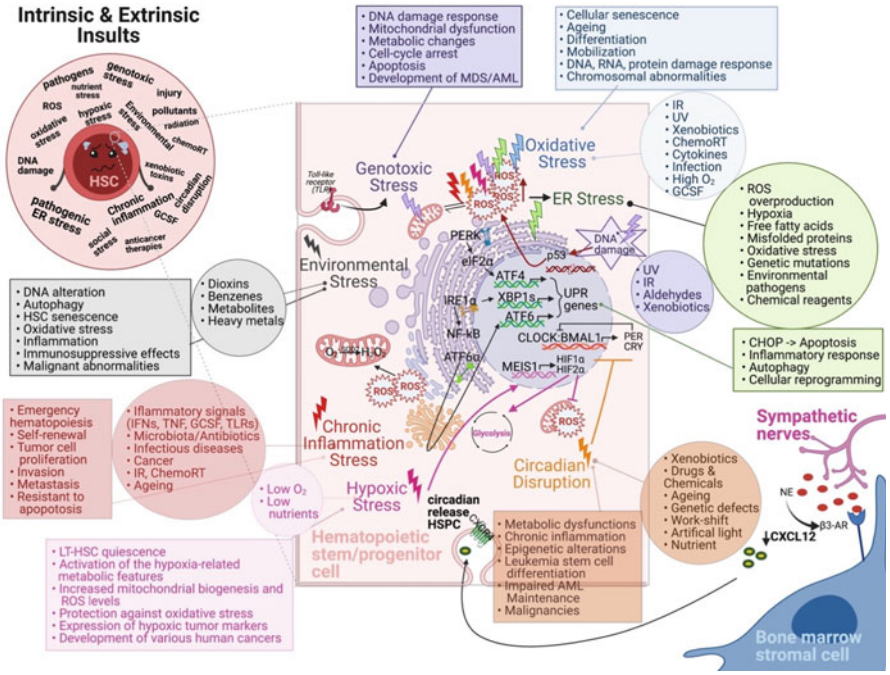

Fig. 1 Summary of the hematopoietic stressors and stress response of the hematopoietic system (Created with BioRender.com)

and reconstitute healthy hematopoiesis. Thus, acute stress of low/medium intensity may be overcome with a limited impact on HSCs. On the other hand, prolonged sustained exposure to stress signals can lead to a shift of HSCs towards HSCs proliferation rather than a quiescent state leading to HSCs exhaustion over time. Chronic inflammation, serial BM transplantation, chemotherapy cycles, genotoxic stress, and aging are conditions leading to disruption of the fine-tuning in the hematopoietic compartment resulting in increased proliferation and HSCs exhaustion. This may lead to the development of cytopenia, aplastic anemia, or upon acquisition of cytogenetic abnormalities in the cycling cells may play a role in the development of hematological malignancies. Recent evidence demonstrates that ablation of inhibitor of DNA binding 1 (Id1) gene can protect HSCs from exhaustion during chronic proliferative stress by promoting HSCs quiescence (Singh et al. 2018). It was shown that Id1 is induced in HSCs by cytokines that promote HSC proliferation and differentiation, suggesting that it functions in stress hematopoiesis and its genetic ablation decreases in HSCs' proliferation, mitochondrial biogenesis, and ROS production. Considering the role of high ROS levels in the BM microenvironment on increased proliferation and differentiation, targeting molecular pathways that reduce ROS in HSCs is suggested as a protective strategy to promote HSC quiescence and limit HSC loss during chronic stress (Singh et al. 2020).

Recent studies have described alterations in the replicative status of the stromal cells as well, similar to HSCs. It was reported that almost all niche cells were quiescent during homeostasis (<1% cycling stromal niche cells). On the other hand, up to 5% of stromal niche cells entered cell cycle 5 days after 5-fluorouracil injection. In addition, a novel sub-cluster of adipocyte-primed LEPR+ cells showing high similarities with Adipo-CARs was identified during hematopoietic stress (Tikhonova et al. 2019; Baccin et al. 2020).

Emergency Hematopoiesis

The kinetics of HSC biology under different stress conditions is an important topic with clinical implications (Lu et al. 2019). The term "emergency hematopoiesis," although mentioned in the 1990s, is better understood by the recently described hematopoiesis scheme and demonstration of heterogeneity within HSCs and progenitor populations and alternative routes of hematopoietic differentiation (Lurie and Danon 1992; Croker et al. 2016; Woolthuis and Park 2016; Niederkorn et al. 2017; Hérault et al. 2017b; Fuchs et al. 2020).

New mechanisms are described in the field of emergency granulopoiesis, highlighting spatiotemporal positioning (Hérault et al. 2017b). Investigation of granulocyte-macrophage progenitors' (GMPs) behavior in mice demonstrated the unique organization of GMPs in the BM. These progenitors proliferate transiently and rapidly in patches, forming condensed, differentiating clusters upon molecular reprogramming. The individual GMPs were scattered throughout the BM in steady state; however, following experimentally induced emergency myelopoiesis, GMPs were organized in loosely collected patches, and then condensed to form distinct, compact, and transient clusters of GMPs surrounded by differentiated myeloid cells. On the other hand, as opposed to transient emergency stress response, in the malignant scenario, such as myeloid leukemia, GMP clusters were constantly produced associated with persistent activation of the self-renewal network and a lack of termination cytokines that usually restore hematopoietic stem cell quiescence.

Different differentiation pathways for thrombopoiesis were also described in the recent years, such as megakaryocyte (MK) generation without transition through the multipotent or bipotent MK-erythrocyte progenitor stage. A subset of HSCs with biased MK potential leading to MK generation was shown to arise directly from HSCs under steady-state and stress conditions (Haas et al. 2015; Nishikii et al. 2017; Jacobsen and Nerlov 2019; Laurenti et al. 2019). Another mechanism reported for emergency platelet release is the MK rupture process instead of the classical method of in vivo platelet generation from MKs through the pro-platelet formation. This mechanism of thrombopoietin (TPO)-independent MK production was shown to occur under inflammatory conditions or after platelet loss associated with acute elevation of IL-1α at the expense of the classical platelet growth factor TPO. It is suggested that the balance between TPO and IL-1α regulates MK cellular programming for thrombopoiesis in response to acute and chronic platelet needs (Nishimura et al. 2015); therefore, it is a vital determinant in stress hematopoiesis. These recent

studies demonstrate that the hematopoiesis scheme changes according to the stress stimulus. The BM microenvironment is critical in the execution of the stress response, e.g., by providing appropriate soluble factors, on this occasion.

Environmental Hematopoietic Stressors

Environmental Pollutants

A critical topic regarding the stress response of BM microenvironment is exposure to environmental stressors, mainly environmental pollutants, which have become a critical topic in the modern era (Scharf et al. 2020). The hematopoietic system is prone to xenobiotic effects, environmental and occupational pollutants, therapeutic molecules, and drugs of abuse. Nutritional status can also directly affect progenitor cells at their differentiation and maturation stages. Such exposures may lead to hematological disturbances, including malignancies. For cancer types with environmental risk factors, important factors in evaluating cancer risk are the route of exposure, the dose, and the duration of exposure. Gene-environment interactions are also important determinant of vulnerability to develop cancer due to environmental factor, exposure, and the risk may depend on a complex interaction between the genetic background and exposure to that particular agent.

Benzene

The best-known pollutant, benzene, has been recognized for several decades for its effects in inducing hematological disorders and leukemia (Cronkite et al. 1989). It is a volatile liquid aromatic hydrocarbon solvent, a byproduct of petroleum refinement accepted as an environmental carcinogen due to its BM toxicity. Detoxification by redox systems in BM, such as NADPH–quinone oxidoreductase1, reduces the local levels of oxidative toxic agents generated by its metabolism. Still, prolonged exposure leads to persistently high levels of benzene and accumulation of its metabolites, inducing toxicity by interfering with different hematopoiesis pathways due to the actions of its multiple metabolites. These metabolites show significant adverse effects on the BM microenvironment: increase ROS generation and inhibit HIF-1 in stem cell niches leading to BM failure presenting as cytopenias, aplastic anemia, and may progress to myelodysplasia and myelogenous leukemia by induction of chromosomal aberrations, gene mutations, oxidative stress, apoptosis, epigenetic deregulation, impairment of DNA repair, modification of protein secretion, and suppression of immune systems (Snyder 2004). In a recent study, transcriptome profiles of C57B/6 mice HSCs following benzene exposure revealed >1500 differentially expressed genes in BM HSCs involved in pathways in cancer, transcriptional mis-regulation in cancer, and hematopoietic cell lineage. Investigations in peripheral blood revealed hematopoietic cell lineage and leukocyte transendothelial migration as critical pathways. Evaluation of co-regulated pathways demonstrated neutrophil degranulation, CD93 (involved in the adhesion, migration, and phagocytosis), and 53 genes that pointed out mechanisms in regulating leukemia stem cell self-renewal and quiescence (Sun et al. 2021). Other studies involving modulation of the cytosolic transcription factor aryl hydrocarbon receptor (AhR), the xenobiotic

response regulator, revealed the important role of AhR in benzene-induced hematotoxicity (Hirabayashi et al. 2004; Scharf et al. 2020).

Nanoparticles and particulate matter

These are other groups of environmental stressors found in polluted air. The degree of toxic systemic effects is closely linked to particle size. Both nanoparticles and particulate matter PM 2.5 (smaller than 2.5 μ m) (Xing et al. 2016) can reach the bloodstream from alveoli and be distributed into tissues, including the BM. The particles can also contain harmful airborne microorganisms and metals. Chronic exposure leads to local and systemic inflammation, may affect HSC niches, and lead to a release of immature granulocytes into circulation (Tan et al. 2000). Recent studies have described the adverse effects of maternal exposure to PM 2.5 during pregnancy on the offspring hematopoiesis (Bhattarai et al. 2020).

Dioxins as Persistent Organic Pollutants (POPs)

POPs are released into the atmosphere as undesired byproducts of an anthropogenic and natural origin. These pollutants can originate as byproducts from combustion processes, such as the incineration of solid waste; the chlorine bleaching of paper and wood pulp; the burning of coal in power plants; forest wildfire; and contaminants in pesticides, herbicides, and fungicides. Dioxins are considered the most hazardous persistent organic pollutants to human health, with their significant toxic effects being linked to binding to AhR in several cell types. AhR is the central regulator of responses to environmental factors and xenobiotic metabolism. The most toxic member of the polychlorinated dibenzodioxins family 2,3,7,8-tetrachlorodibenzodioxin (TCDD) can bind to AhR, leading to several toxic effects. Many stressors can drive AhR activation and nuclear translocation, leading to increased expression of target genes (e.g., cytochrome P450; CYP1A1, CYP1A2, CYP1B1) (Safe et al. 2018). AhR is also a crucial homeostasis modulator in several tissues and biological processes, including hematopoiesis. Epidemiologic studies have demonstrated associations between TCDD and hematological malignancies such as non-Hodgkin lymphomas, chronic lymphocytic leukemia, and multiple myeloma (Bertazzi et al. 1993; Fracchiolla et al. 2016). It has been reported that the BM of adult mice exposed to acute doses of TCDD becomes hypocellular, with a significant decrease in the total number of HSCs due to AhR modulation (Ahrenhoerster et al. 2014).

Polycyclic Aromatic Hydrocarbons (PAH)

These are ubiquitous environmental pollutants that include over 200 compounds with two or more fused benzene rings. These compounds are formed due to incomplete combustion of fossil fuels, are created in the car and diesel exhaust, and are formed in smoked or charbroiled food. They are also found in cigarette smoke condensate and tobacco products. Studies have demonstrated an association between exposure to PAHs and cancer initiation and progression. It was shown that PAHs must be metabolically activated to reactivate genotoxins to cause their mutagenic and carcinogenic effects. A recent study described activation of the AhR/CYP1A pathway and epigenetic regulation of cancer stem cells upon PAH

exposure (Akhtar et al. 2020). Moreover, adverse hematopoietic effects of occupational PAH exposure were shown, and oxidative stress-mediated toxicity was suggested in another recent study (Cao et al. 2020).

Heavy Metal Exposure

This is also associated with hematopoietic disorders. Industrial and urban growth has increased the risk of exposure of humans to metals. Heavy metals may cause alterations in the BM, causing anemia, immune deficiency, coagulation defects, and may be associated with leukemia development. Among heavy metals, lead, mercury, cadmium, and arsenic have been associated with hematological disorders (dos Vianna et al. 2019). It has been shown that lead exposure even at low levels in humans under occupational exposures is toxic to the BM and leads to reduced colony-forming capacity; arsenic trioxide administered to mice severely damages the BMME and hampers the formation of a healthy matrix to support hematopoietic progenitors; mercury exposure is associated with hematopoietic disturbance characterized by anemia and lymphocytopenia; and cadmium exposure leading to increased myelopoiesis and suppressed lymphopoiesis (Zhang et al. 2016b; Medina et al. 2017; Pereira and Law 2018).

Detoxification Response and Signaling Pathways

Drug metabolizing enzymes Cytochrome P450 (CYP) and Glutathione S-Transferases (GSTs)

Drug metabolizing enzymes display a high degree of polymorphism in the general population. Functional polymorphisms in the genes encoding xenobiotic-metabolizing enzymes result in interindividual differences that determine the effects of toxic exposures (Harris 1989). Cytochrome P450 (CYP) and glutathione S-transferases (GSTs) are the main detoxification pathways. The latter catalyzes the conjugation of the reduced form of glutathione to xenobiotic substrates to detoxify cellular environments. Cytochrome P450 family proteins are the major enzymes involved in drug metabolism and metabolize thousands of endogenous and exogenous chemicals. In addition, they metabolize endogenous and exogenous DNA-reactive chemical compounds and xenobiotics (Zanger et al. 2004).

Aryl Hydrocarbon Receptor (AhR)

AhR is the central regulator of responses to environmental factors and of xenobiotic metabolism, which was initially described in dioxin toxicity (TCDD; 2,3,7,8-Tetrachlorodibenzo-P-dioxin) (Poland et al. 1976), now is accepted as a crucial regulator of homeostasis in several tissues/systems in the body. There are many endogenous ligands of AhR. The AhR-signaling acts as a xenobiotic sensor regulating drug-metabolizing enzymes of the cytochrome p450 family. Various structurally diverse compounds of the environment, chemicals, toxicants, pollutants, diet, microbiome, and host metabolism can induce AhR activity (Mandal 2005; Vogel et al. 2020). Under homeostatic conditions, AhR remains predominantly in the cytoplasm as part of a protein complex linked to molecular chaperone heat shock protein 90 (HSP90),

p23, and XAP. Several stressors can drive AhR activation and evoke its conformational transition, resulting in its nuclear translocation. AhR then dissociates from HSP90 and binds to the AhR nuclear translocator (ARNT) The AhR/ARNT complex binds to promoter regions in the DNA known as AhR-responsive DNA elements or xenobiotic response elements (XREs), which leads to an increased expression of target genes (e.g., CYP 1A1, CYP1A2, and CYP1B1). This canonical pathway for AhR activation mediates several toxic responses, including liver damage, chloracne, teratogenesis, cancer, and immunosuppression (Barouki et al. 2007; Fujii-Kuriyama and Kawajiri 2010; Furue and Tsuji 2019).

In addition to exogenous triggers, many endogenous ligands have been shown to induce AhR-signaling, with implications on several body systems playing a critical role in balancing physiological functions. For example, the significant effects of AhR signaling on circadian clock genes also pointed out the potential impact of different neurotransmitters and metabolites (Kou and Dai 2021). The AhR modulation has shown significant effects on hematopoiesis. In addition, it has been demonstrated that HSCs from AhR knockout mice leave quiescence and become hyperproliferative, suggesting a role for AhR in negative regulation of excessive proliferation (Singh et al. 2009, 2011).

Toll-like receptor (TLR) Signaling

Toll-like receptor signaling is needed in responding to inflammatory stressors, microbial agents and is required for innate and adaptive immunity. Pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) are released in response to injury and stimulate TLR-signaling. In addition to their well-known role in the innate immune response to acute infection or injury, accumulating evidence supports a role for TLRs in the development of hematopoietic and other malignancies. HSCs proliferate in response to PAMPs and DAMPs through TLRs, or in response to pro-inflammatory cytokines through receptors expressed on HSCs (Schuettpelz and Link 2013; Mirantes et al. 2014). Acute stress promotes HSCs' proliferation and differentiation that is quickly resolved, whereas prolonged or chronic stress can lead to HSC exhaustion (BMT, chronic infection/inflammation, chemotherapy) (Singh et al. 2020). Several hematopoietic disorders, including lymphoproliferative disorders and myelodysplastic syndromes, which possess a high risk of transformation to leukemia, have been linked to aberrant TLR signaling. Furthermore, activation of TLRs leads to the induction of several pro-inflammatory cytokines and chemokines, which can promote tumorigenesis by driving cell proliferation and migration and providing a favorable microenvironment for tumor cells (Monlish et al. 2016).

BM Microenvironment Alterations, Dysregulation, and Myeloid Leukemia

Due to close interaction between the hematopoietic compartment and the microenvironment, several nonmalignant and malignant conditions are frequently associated with alterations in the BM microenvironment composition and function (Fig. 2).

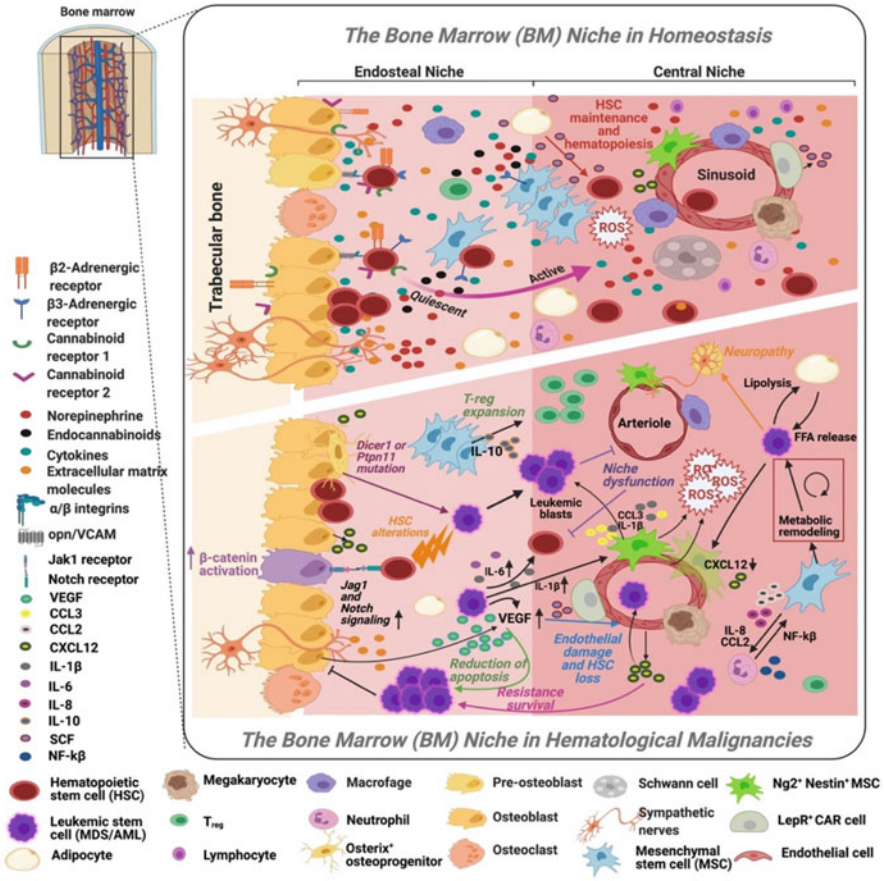

Fig. 2 Summary of the bone marrow niche structure and organization and its role in homeostasis and in hematological malignancies. (Created with BioRender.com)

Leukemia results from a clonal transformation of hematopoietic precursors by acquiring chromosomal rearrangements, genetic mutations, and epigenetic modifications. It has been shown that the initiating intrinsic event leads to alterations in the BM microenvironment to support the survival of leukemic blasts (Blau et al. 2007; Li and Calvi 2017). There is limited data for a driver role for the BM microenvironment, leading to the development of leukemia. It has been suggested that prolonged sustained exposure to stress signals, including chronic inflammation, chemotherapy, environmental toxins induced alterations in the BM microenvironment leading to increased proliferation, and HSCs' exhaustion over time, resulting in BM dysfunction, failure, or development of hematological malignancy, usually of myeloid lineage (Cho et al. 2020).

Significant changes are detected in the BM microenvironment in hematological malignancies, during the course or before the development of leukemia (Dührsen and Hossfeld 1996). Various alterations involving MSCs, osteoprogenitors, adipocytes,

endothelial cells, ECM, and soluble factors have been reported in the leukemic niche playing significant roles in initiation, progression, and chemoresistance.

The majority of studies performed on MSCs described alterations in their adhesive, proliferative, and secretory characteristics. Reduced HSCs' maintenance factors including CXCL12, KİT ligand, Ang1 were described in leukemic MSCs (Chen et al. 2016). Further studies have shown increased expression of pro-inflammatory molecules such as TNF, IL6, C-C motif chemokine ligand 3 (CCL3), alterations in SNS signaling due to damaged tyrosine hydroxylase sympathetic nerve fibers, altered stem cell trafficking, and dysregulated vascularization in the leukemic BM microenvironment (Maryanovich et al. 2018; Méndez-Ferrer et al. 2020).

In humans, the development of myelodysplastic syndrome (MDS)/secondary acute myeloblastic leukemia (sAML) years after chemotherapy use, besides intrinsic factors, emphasizes the active role of the altered and dysregulated microenvironment in leukemogenesis (Sperling et al. 2017). It may be speculated that, in both situations, the BM microenvironment alterations lead to dysregulation in time, playing a critical role in the progression towards malignancy and evolution to AML after a myelodysplastic stage (e.g., 5–7 years after alkylating agent exposure) or without such dysplasia (e.g., 2–3 years after topoisomerase II inhibitor exposure) (Raaijmakers et al. 2010; Duarte et al. 2018; Forte et al. 2019). Apart from those toxic exposures or cancer-predisposing disease, various alterations have been reported in the BM microenvironment that may be closely associated with the leukemogenic process (Ciciarello et al. 2019; Kazianka and Staber 2020). Understanding the mechanisms leading to the development of sAML may help identify the microenvironmental factors in the BM.

Development of Secondary Myeloid Malignancy

Unlike *de-novo* AML, in which there is a lack of a typical clinical history, sAML occurs following environmental or toxic exposures and/or develops in patients with an antecedent hematological disorder; therefore, it represents a model to study alterations in the BM microenvironment that may participate in leukemogenesis. It has been shown that inherited diseases (e.g., Fanconi anemia (FA), Shwachman-Diamond syndrome (SDS)) (Horwitz 1997) and/or environmental exposures (e.g., high-level ionizing radiation, cigarette smoking, long-term occupational exposure to benzene, exposure to certain chemotherapy drugs such as alkylating and platinum agents, epipodophyllotoxins, immunosuppressives, and radiation therapy) may lead to leukemia development in some cases of AML (Daniels et al. 2013; Radivoyevitch et al. 2016; Matsuo et al. 2016). The incidence of sAML is rising in parallel with the increasing number of cancer survivors. Its prognosis is poor compared to *de novo* AML, which occurs without previous therapy or without the antecedent disease.

Therapy-Related Myeloid Malignancy

Secondary AML is further divided into therapy-related AML (t-AML) with previous exposure to chemotherapy and radiotherapy, or AML evolving from antecedent

hematological disorder including MDS, aplastic anemia, or chronic myeloproliferative disorders. Therapy-related myeloid neoplasms (t-MNs), including AML, occur as late complications of cytotoxic chemotherapy and/or radiation therapy and patients show high-risk features. Lymphoma and breast cancer are the most common primary malignancies associated with t-AML. Patients with an inherited predisposition such as mutations in DNA damage sensing or repair genes or polymorphisms in genes that affect drug metabolism or transport are at higher risk for developing t-MNs (Vardiman et al. 2002; Seedhouse et al. 2004; Larson 2009; Bhatia 2013). A wide variety of agents with different mechanisms of action are associated with the development of t-MNs. The interval between treatment and the outcome of the disease varies according to the type of therapy. In patients exposed to alkylating agents, t-AML is usually preceded by myelodysplastic changes and the latency period is long (5–7 years).

Contrarily, in patients previously treated with topoisomerase II inhibitors, the latency period is shorter (2–3 years) than alkylating agent exposure, is not associated with prior MDS, and exhibits rapidly progressive leukemia. These patients usually present with balanced translocations involving 11q23 and 21q22 abnormalities. These agents stabilize the enzyme-DNA covalent intermediate, decrease the religation rate, and cause chromosomal breakage. Repair of chromosomal damage results in chromosomal translocations, leading to leukemogenesis (Bhatia 2013; Tiruneh et al. 2020).

Increasing knowledge on the pathophysiology of t-AML may help identification of the contributing microenvironment factors. Among other mechanisms involved in developing t-AML, abnormal p53 activity leads to increased susceptibility to leukemogenesis manifesting with reduced ability to repair DNA damage and genomic instability. Telomere shortening also contributes to t-MDS/AML by limiting hematopoietic proliferation and regenerative capacity and inducing genetic instability. Following genotoxic exposure, the increased replicative demand on hematopoietic cells associated with hematopoietic regeneration can lead to accelerated telomere shortening. On the other hand, some polymorphisms in the MDR1 gene are suggested as risk factors for t-MDS/AML (Bhatia 2013; Tiruneh et al. 2020).

An important mechanism implicated in t-AML development includes defects of the individual DNA repair machinery. Double-strand breaks in DNA following ionizing irradiation and chemotherapy exposure may lead to cell death or loss of genetic material, resulting in chromosome aberrations. Unsatisfactory repair results in the acquisition and persistence of mutations. On the other hand, better repair mechanism will inhibit apoptosis, enabling the survival of a cell with damaged or poorly repaired DNA (Leone et al. 2007). Evidence suggests a role for mismatch repair in susceptibility to t-AML reflected as microsatellite instability (Seedhouse et al. 2004). Other mechanisms involved include the base excision repair pathway, which corrects individually damaged bases, occurring as the result of endogenous processes, ionizing irradiation, and exogenous xenobiotic exposure; and nucleotide excision repair that removes structurally unrelated bulky damage induced by ultraviolet radiation, environmental factors, and endogenous processes, and repairs a significant amount of DNA damage caused by chemotherapeutic agents.

Detoxification pathways play a critical role in the pathophysiology of malignancy. Polymorphisms are frequent in drug-metabolizing enzymes. It is suggested that polymorphisms in detoxification enzyme and DNA repair genes synergistically may affect the risk of AML, such as in patients with deletion of the GSTM1 gene and with increased DNA damage (Seedhouse et al. 2004). Glutathione S-transferases are involved in the detoxification of potentially mutagenic and DNA-toxic metabolites of several chemotherapeutic agents. Accumulation of reactive species that escape detoxification mechanisms or are produced in excess due to drug-metabolizing enzyme polymorphisms, together with impaired DNA damage repair, are predisposing factors to t-AML (Davies et al. 2001; Hatagima 2002; Seedhouse et al. 2004). Polymorphisms in cytochrome P450 family compounds are also implicated in leukemia development and prognosis. AhR activation by external and endogenous stressors and regulation of the CYP family of drug-metabolizing enzymes is essential to maintain homeostasis in the hematopoietic system. A recent study has shown increased quiescence in AML stem cells upon suppression of AhR signaling (Ly et al. 2019). In another study, the AHR pathway was activated in human and murine AML and impaired natural killer cell development and function through regulation of miR-29b expression. This effect was reversed with the AHR antagonist pointing out the clinical implications of AhR modulation as a therapeutic strategy in cancer (Scoville et al. 2018). AhR-mediated response has been reported to have a critical role in benzene-induced AML as well; however, the involvement of CYP1A1 and other CYP compounds is not apparent in benzene-induced leukemia (Yoon et al. 2002). On the other hand, *CYP3A* was shown to participate in the metabolism of various chemotherapeutics, including topoisomerase II inhibitors and alkylating agents and polymorphisms associated with t-AML. A recent report suggested the favorable role of CYP3A inhibitors in patients with AML carrying FLT3/internal tandem duplication (ITD) mutations through the reversal of the protective effect mediated by the BM microenvironment against FLT3 tyrosine kinase inhibitors (Chang et al. 2019). It was reported that BM stromal cells express CYPs, including CYP3A4, and inhibit the activity of tyrosine kinase inhibitors. Clarithromycin, a clinically active CYP3A4 inhibitor, antibiotic was effective in overcoming BM microenvironment-mediated drug resistance.

Toll-like receptor signaling has also been implicated in leukemia. In addition to mediating innate immune system response to acute infection or injury, the TLR signaling pathway was also shown to play a role in developing hematological malignancies by inducing the establishment of a pro-inflammatory tumorigenic environment (Monlish et al. 2016; Singh et al. 2020).

Leukemia-Predisposing Inherited BM Failure Syndromes

Fanconi anemia

Fanconi anemia (FA) is discussed here as a model disease to evaluate myeloid leukemia development both intrinsic and extrinsically due to the genetic defect in DNA damage repair mechanisms and extreme sensitivity to toxic and environmental agents.

It is an inherited disease characterized by congenital abnormalities, BM failure, and cancer predisposition manifesting as myeloid malignancy (AML, MDS) and solid tumors. The condition is caused by mutations of the Fanconi anemia/breast cancer (FA/BRCA) pathway, critical in cellular processes and DNA repair interstrand crosslink (ICL). Genomic instability, cell death, alterations in cell cycle, extreme sensitivity to DNA cross-linking agents (cisplatin, mitomycin C, diepoxybutane, endogenous aldehydes), and oxidative damage are the typical cellular, molecular events. Two DNA strands are covalently joined, DNA replication and transcription are impaired, resulting in the accumulation of toxic DNA double-strand breaks. Two ICL repair mechanisms are present. One mechanism, replication dependent, requires the FANC proteins and the other one operates outside the S phase, involves nucleotide excision repair (Houghtaling et al. 2005). FA proteins have roles in the sensing, recognition, and processing of ICLs. To repair this type of DNA damage, alternative error-prone pathways of DNA ICL repair become activated, leading to the formation of gross structural chromosome aberrations, breakage, translocations, dicentrics, and acentric fragments, and the development of hematological abnormalities and leukemia (Garaycoechea et al. 2018).

An essential feature of the disease is sensitivity to aldehydes, which cause various DNA lesions, including ICLs and DNA protein cross-links. Humans are exposed to endogenous and exogenous sources of ICL-inducing agents, and most of this damage is successfully repaired by the FA/BRCA pathway. Reactive aldehydes, the byproducts of normal cellular metabolism, are important genotoxins neutralized by the FA/BRCA pathway. Therefore, the exposure of FA deficient cells to aldehydes results in the accumulation of chromosomal aberrations, including the stem and progenitor cell populations with aldehyde susceptibility. Aldehyde dehydrogenase enzymes are required for aldehyde detoxification, and polymorphisms may affect the phenotype of FA patients (Kim and D'Andrea 2012; Garaycoechea et al. 2012, 2018; Ceccaldi et al. 2016).

Several mechanisms lie under hematological abnormalities of FA. p53 hyperactivation is an important mechanism responsible for BM failure. BM cells exposed to replicative stress during prenatal HSC expansion trigger an apoptotic p53/p21-mediated response leading to a prenatally reduced fraction of CD34+ cells and a compromised HSC pool. In the postnatal period, DNA damage accumulation contributes to BM failure (Ceccaldi et al. 2012). In addition to p53, there is hyperactivation of the potent growth inhibitory TGFβ pathway (Zhang et al. 2016a). Compensatory overexpression of the MYC oncogene in a subset of HSCs may further increase their replicative stress (Rodríguez et al. 2021). Another mechanism of hematological defects is overexpression of cell cycle regulating ATM and CHK1 kinases in FA cells leading to cell cycle arrest in basal conditions (García-De-teresa et al. 2020).

The risk of AML, MDS is significantly increased, necessitating periodic BM investigations. Clonal evolution in the BM occurs in 40% of children and young adults with FA (Bagby and Fleischman 2011). The presence of clonal and non-clonal chromosomal alterations both are valuable biomarkers for detecting progression to cancer.

Recent studies have focused on identifying of the defects in the BM microenvironment of patients with FA that may contribute to hematological disturbances and malignancy. MSCs, giving rise to cells of stromal members including osteoblasts, adipocytes, and chondrocytes (Caplan 1991), are the essential cellular components of the BM microenvironment; hence, they have been investigated in single-cell, co-hepa studies or in advanced engineered systems to understand the BM niches in healthy and diseased states. Other investigators and we studied the characteristics of BM-MSCs in patients with FA. Although the standard phenotypic and functional characteristics showed similarities to healthy controls, several alterations were detected, including decreased CFU-F ability, proliferative capacity, early senescence, and spontaneous chromosomal fragility (Mantelli et al. 2015).

Our studies also showed decreased proliferation, increased ROS levels, and an arrest in G2 upon mutagen stimulation, a lack of TGF-beta synthesis, and early senescence in FANCD2-deficient BM-MSCs (Cagnan et al. 2018). In addition, significant downregulation of the TALE class member PKNOX2 was detected in FA MSSCs. TGF-β1 stimulation increased PKNOX2 expression suggesting an association with disease pathophysiology (Cagnan et al. 2019). In another study, the functional defects in HSC differentiation were attributed to the effects of MSC-glycerolipids on TLR signaling (Amarachintha et al. 2015). The results of these studies suggest the presence of a dysregulated BM microenvironment in FA contributing to the hematological defects presenting as cytopenia, BM failure, and/or leukemia. These findings observed in FA BM-MSCs necessitates further investigations on whether the BM microenvironment has a primary or a significant role in the leukemogenesis process.

Shwachman-Diamond syndrome

Shwachman-Diamond syndrome is another inherited BM failure caused by mutations in the Sbds gene, which encodes a protein involved in ribosomal maturation. The disease is characterized by skeletal defects, pancreatic insufficiency, neutropenia, and a high probability of developing into MDS and AML. Experimental studies showing the leukemia-driver role of Sbds modifications in the stromal compartments of the BM have been significant achievements towards understanding the development of leukemia. Conditional deletion of the RNA-processing endonuclease enzyme dicer1 in primitive osterix-expressing osteolineage cells was shown to lead the development of MDS/AML in mice demonstrating the role of a niche-driven mechanism mice (Raaijmakers et al. 2010). The loss of dicer1 resulted decreased ribosome maturation factor Sbds expression in mesenchymal/osteoprogenitor cells. In further experiments, niche alteration led to increased p53 levels and secretion of the pro-inflammatory factors (DAMP genes S100A8 and S100A9) that resulted in mitochondrial dysfunction in mouse and human HSC, and progenitors through binding to TLR-4. The authors suggested that activation of the p53-S100A8/9 axis in BM-MSCs has a predictive value in MDS progression (Zambetti et al. 2016). This study showed that increased inflammatory signals in the microenvironment caused genotoxic stress in experimental mouse model and SDS patients. The recent description of increased inflammatory findings in patients

with SDS (Furutani et al. 2020) further emphasizes the necessity for close follow-up of patients with BM examinations. It brings out the issue of BM microenvironment evaluations to the clinics. However, this area is new, and clinical translation needs further investigations on this topic.

BM Microenvironment *as a Contributor/Driver of Leukemia*

Investigation of human BM samples obtained after diagnosis of leukemia is not a suitable tool to study niche-driven mechanisms since microenvironmental changes that precede the diagnosis of the disease cannot be detected. Development of donor-derived leukemia after allogeneic BM transplantation with a different clone than the patient's one suggests that the BMME alterations induced by the effects of the conditioning regimen and the transplant process might have played an essential role in leukemic transformation (Wiseman 2011). In vivo experimental niche modifications towards leukemia induction have provided valuable data and provided evidence for a leukemia-driver role for the stroma. These investigations on knockout and conditional deletion models have shown the direct contribution of stromal defects to myeloid leukemia development pointed out the critical roles of different mechanisms involving retinoic acid receptor gamma, the RNA-processing endonuclease enzyme dicer1, the ribosome maturation factor Sbds, and protein tyrosine phosphatase non-receptor type11 and 21 (PTPN11, PTPN21) (Walkley et al. 2007; Garcia and Chen 2017; Alter 2017; Kokkaliaris and Scadden 2020). The stromal cell type plays a role in the leukemogenic process. It was shown that conditional deletion of primitive osterix (Osx)-expressing osteolineage cells, but not mature osteoblasts, led to MDS/AML in mice (Raaijmakers et al. 2010). Our investigations on human BM-MSCs, the precursors of osteolineage cells, supported these findings showing differential expression of dicer1 and differences in microRNA profiles among patients with MDS, AML, and healthy controls (Ozdogan et al. 2017).

Moreover, activating mutations of PTPN11 in mice MSCs and osteoprogenitors lead to the development of a myeloproliferative, juvenile myelomonocytic leukemia-like disease as observed in patients with Noonan syndrome carrying this mutation. Similarly, overexpression of PTPN21 in BM-MSCs of acute lymphoblastic leukemia (ALL) patients was associated with alterations in MSCs differentiation and immunomodulatory characteristics (Wang et al. 2019). PTPN21 was shown to have a role in regulating cytoskeleton-associated cellular processes (Carlucci et al. 2008), and overexpression in MSCs leads to an acceleration of leukemic and endothelial cell recruitment leukemic cell proliferation and drug resistance (Wang et al. 2019). However, when knocked-down on HSCs ($Ptpn21^{-/-}$), the cell stiffness was lost, cells gained deformability and mobility (Ni et al. 2019). Another study also provides evidence for a highly active/initiator role for osteoblasts in the leukemogenic process. When a constitutively active form of b-catenin was expressed on osteoblasts, the differentiation program of HSCs was shifted towards myeloid lineage through upregulation of the Notch pathway leading to AML development (Garcia and Chen 2017).

The majority of studies on BM niche interactions consist of studies on MSCs and osteoblastic lineage cells. The highly active roles of adipocytes and endothelial cells are better defined in recent studies. Marrow adipose tissue has an essential role in developing leukemia and in BM metastasis of solid tumors (Shafat et al. 2017; Kumar et al. 2020; Liu et al. 2020a). However, these studies describe the remodeling of adipocytes by malignant cells, which then significantly affect cancer cell behavior. Endothelial cell modifications are also implicated in leukemogenesis. It has been shown that loss of canonical Notch signaling in endothelial cells leads to constitutive activation of mir-155 and NF-kB signaling, increased levels of pro-inflammatory cytokines (GCSF, TNFα) resulting in expansion of immature myeloid cells and a myeloproliferative-like disease. Another example of endothelial cell involvement in the direct leukemogenesis process is which also deletion of signal-induced proliferation-associated gene 1 (Sipa1) in endothelial cells also resulted in myeloproliferative neoplasms (Fernandez et al. 2008; Wang et al. 2014; Xiao et al. 2018).

Expansion of the myeloid compartment of the BM, clonal hematopoiesis, and chronic immune stimulation are risk factors for the development of MDS/AML, which are findings observed in aging hematopoiesis (Kristinsson et al. 2011; Yoshizato et al. 2015). Aging is associated with lymphoid suppression and myeloid skewing attributed to high inflammatory cytokine rantes (CCL5) in the BM microenvironment. In a heterochronic setting, it was shown that aged HSCs placed in a young environment generate fewer myeloid cells providing (Ergen et al. 2012) evidence for a critical role for environmental factors in establishing age-associated hematological defects, including malignancy. The spatial distribution of HSCs in aging BM niches has shown differences by localizing away from the endosteum, potentially hampering the ability of HSCs to remain quiescent and leading to a decreased pool of primitive HSCs (Ho et al. 2019). Considering the increased risk of myeloproliferative diseases, MDS, and AML in the elderly, the myeloproliferative, immunosuppressed, adipogenic, and chronic inflammatory state of the dysregulated BM microenvironment points out its essential role in malignant transformation.

Exposure of the BM microenvironment to exogenous and endogenous stressors is associated with the induction of a pro-inflammatory state. Among these, GCSF will be addressed due to its clinical implications and frequent use. Patients with BM failure suffering from chronic cytopenia may be exposed to chronic use of GCSF. Spatial positioning in the BM niches is important in determining stem cell fate. GCSF has gross effects on the BM microenvironment, leading to increased cell trafficking towards the sinusoidal niche. It was shown that upon transmigration of HSCs from the endosteal to the sinusoidal niche, they proliferate and become sensitive to genotoxic stress induced by irradiation or myeloablation. GCSF removes HSCs from the endosteal niche where they are protected from toxic insults, thus increasing their susceptibility towards genotoxicity. Therefore, chronic administration of GCSF in congenital neutropenias has been a concern raising the risk of MDS/AML, particularly in patients with SDS with and in those with Ras or GCSF receptor mutations necessitating special scheduling and dosing recommendations for patients on long-term use of GCSF (Freedman and Alter 2002). Alternatively, GCSF is mainly used in some chemotherapy protocols as an adjunct treatment strategy to

induce mobilization of leukemic cells from their quiescent niches (Lapidot et al. 2007) and render them vulnerable to cytotoxic or pro-apoptotic chemotherapy (de la Rubia et al. 2002).

Spatial positioning of HSCs and leukemic stem cells is critical in disease pathophysiology. In addition to inducing HSC mobilization, SNS signals are essential for the regeneration of hematopoiesis following genotoxic stress (Lucas et al. 2013). Exposure to neurotoxic agents such as chemotherapy or irradiation was shown to damage the BMME leading to sympathetic neuropathy associated with MSC and endothelial cell proliferation, further sensitization towards genotoxic insults leading to a reduced niche size and failure to support hematopoietic recovery. Studies revealed that AML disrupts the SNS nerves and the quiescence of Nestin^{+} niche cells. Interestingly, SNS neuropathy is also involved in the promoting leukemic BM infiltration (Hanoun et al. 2014). In the leukemic stroma, adrenergic signaling maintaining niche quiescence was shown to be transduced by the β3 adrenergic receptor as opposed to β2 receptors in the healthy niche regulating osteoblasts. These findings demonstrate the important role of sympathetic neuropathy in niche dysregulation. GCSF use is also associated with involvement of both β2 and β3 adrenergic receptors, pointing out the contributory role of GCSF in the development of a dysregulated BM microenvironment (Takeda et al. 2002; Elefteriou et al. 2005; Méndez-Ferrer et al. 2010a; Arranz et al. 2014; Hanoun et al. 2014; Man et al. 2021). Among several molecules implicated in BM microenvironment cell trafficking and spatial location, the transcription factor Twist1 has been shown to play a newly described role that may have implications in AML pathophysiology. Its deficiency induces cycling and mobilization of HSCs by inhibition of retention factors, stimulation of GCSF expression on stromal cells leading to myeloid proliferation (Niu and Cancelas 2018).

Another issue regarding the role of BM microenvironment in leukemia is the development of drug resistance executed through several mechanisms, including the establishment of multidrug resistance (MDR) phenotype by increased expression of ABC transporters, MSC release of soluble factors, ECVs, the establishment of tunneling nanotubes and mitochondria transfer (Griessinger et al. 2017). The BM-MSCs are highly involved in regulating immune interactions, and MSC-mediated immune modulation/suppression could contribute to tumor progression and drug resistance within the BM niche. Additionally, MSCs may give nutritional support to leukemic cells by synthesizing enzymes (e.g., asparagine synthetase) to provide macromolecules, such as asparagine, conferring protection to leukemic cells from chemotherapy toxicity (Iwamoto et al. 2007). Leukemic lymphoblasts are very sensitive to the depletion of exogenous asparagine and glutamine because of their low capacity to produce their asparagine supply, and the chemotherapeutic agent asparaginase depletes these amino acids. But MSCs, by increasing synthesis, lead to chemotherapy resistance. It has been shown that there is a complex network of metabolic interactions involving malignant cells and their neighbors in the tumor microenvironment and cancer cells could induce stromal cells to produce metabolites and nutrients to support their metabolism, among which glutamine is essential in sustaining the metabolism of proliferating cells and regulating redox homeostasis

in leukemic cells in the hypoxic environment. It was demonstrated that AML blasts utilize glutamine as an alternative carbon source for energy production and are highly dependent on glutamine for proliferation and survival (Kokkaliaris and Scadden 2020).

Metabolic Dysregulation and the Role of Adipocytes in the Malignant Niche

Metabolic factors and nutrient status are increasingly being recognized in cancer pathophysiology, pointing out the critical roles of dietary manipulation. Cancer cells consume high glucose levels, and the majority of them prefer glycolysis even in the presence of oxygen. On the other hand, the Krebs cycle utilizes substrates such as glutamine and fatty acids to generate intermediates for biosynthetic pathways and counteract oxidative stress. The metabolic stress sensor, checkpoint kinase AMP-activated protein kinase is implicated in protecting leukemia-initiating cells from oxidative stress and promoting leukemogenesis. Interestingly, leukemogenesis was profoundly suppressed when associated with dietary restriction (Saito et al. 2015). Studies have shown downregulation of nutrient-signaling pathways by dietary restriction of calories or macronutrients. The main pathway affected is the insulin/insulin-like growth factor 1 (IGF-1) system and its effectors, ERK, MAPK, and PI3K, known to modulate cell survival and proliferation pathways (Lu and Ashraf 2012; Longo and Mattson 2014; Klement and Fink 2016). These studies have led to consideration of dietary restriction and/or the use of insulin-lowering drugs to support cancer therapy.

Fasting leads to significant alterations in lipid metabolism. Lipolysis rates are precisely regulated through hormonal and biochemical signals. In acute conditions, catecholamines bind to β adrenergic receptors on adipocytes and induce triacylglycerol hydrolysis, releasing fatty acids and glycerol that act as oxidative substrates to maintain energy requirements for other metabolic tissues. Chronic exposure to extreme nutritional states, such as obesity or starvation, also induces metabolic adaptations that include changes in lipolysis. Lipolytic products of adipocytes act as signaling molecules regulating metabolic processes in many non-adipose tissues (Yang and Mottillo 2020). Therefore, in addition to its roles in type 2 diabetes, fatty liver disease, and obesity, adipocyte lipolysis is a therapeutic target in malignancy (Munir et al. 2019; Koundouros and Poulogiannis 2020).

Adipocytes have been implicated in favor of cancer cell survival, proliferation, and metastasis in solid tumors, such as breast, prostate, and ovarian cancers. Upon close interaction with malignant cells, these cancer-associated adipocytes undergo alterations and transport lipids to neighboring cancer cells to support tumor growth. Specialized transporters, fatty acid-binding protein (FABP4), fatty acid translocase (FAT/CD36), and fatty acid transport protein (FATP) have been identified as key proteins involved in this mechanism (Dirat et al. 2011). Cancer cells need ATP and macromolecules for proliferation and survival, among which fatty acids play a critical role in membrane biogenesis, energy production, and protein modification.

Thus, lipolysis is an important metabolic process needed for cancer cell survival. Additionally, cancer-associated adipocytes, by releasing adipokines including leptin, adiponectin, IL6, CCL2, and CCL5, were shown to induce proliferation, angiogenesis, dissemination, invasion, and metastasis of breast cancer cell (Zhao et al. 2020). Cancer microenvironment is usually hypoxic with nutrient deficiency. To thrive under these changing and challenging conditions, cancer cells adapt their metabolism. Upon exogenous uptake of fatty acids from surrounding environment, cancer cells can perform *de novo* lipogenesis through which carbon atoms derived from carbohydrates such as glucose and amino acids including glutamine are converted into fatty acids. In normal tissues, de novo lipogenesis is restricted to hepatocytes and adipocytes; however, cancer cells may also reactivate this anabolic pathway. Thus, lipid metabolism remodeling is a metabolic hallmark of cancer cells and consists of alterations in fatty acid transport, de novo lipogenesis, storage as lipid droplets and β-oxidation for ATP generation (Koundouros and Poulogiannis 2020).

Marrow Adipose Tissue and Leukemia

The solid tumor studies demonstrating the involvement of adipocytes in cancer pathophysiology in tissues rich in adipocytes, such as breast, prostate, and ovarian cancers, have reported the role of BM adipose tissue (MAT) in leukemia. The BM is another fat-rich tissue consisting of a unique type of fat (Fig. 3).

The MAT has been a topic of interest in recent years due to its critical role in bone and BM metastasis of solid tumors, and the pathophysiology of hematological malignancies. It is also involved in metabolism regarding obesity, insulin resistance, metabolic syndrome, and cardiovascular defects. MAT is regarded as neither a white nor a brown adipose tissue carrying properties of both, thus suggesting it as a beige adipose tissue, or the fourth type of adipose tissue possessing some characteristics of each depending on the environmental signals. Despite increasing number of studies in MAT, there are many issues to be resolved. For example, there is a lack of standardized criteria for the definition, isolation, and characterization of MAT. Given the clinical implications and the uncertainty in definition and protocols involved therein, the International Bone Marrow Adiposity Society founded a working group to evaluate methodologies in BM adiposity research. Following their annual meeting in 2017, an article about reporting guidelines, review of methodological standards and challenges towards harmonization in BM adiposity research was published in 2020 (Tratwal et al. 2020). These developments point out the need for more studies on the BM microenvironment to understand the role of MAT in the pathogenesis of hematological disorders and pave the way for targeted therapies.

In humans, in some studies, BM adipocytes were isolated by enzymatic digestion of specimens obtained during hip replacement surgery (Attané et al. 2020), whereas in others, *in vitro* differentiated adipocytes from MSCs were regarded as MAT. On the other hand, the fatty tissue of the BM obtained from the femur/tibia was used for isolation of marrow adipocytes in in vivo experimental models (Fazeli et al. 2013;

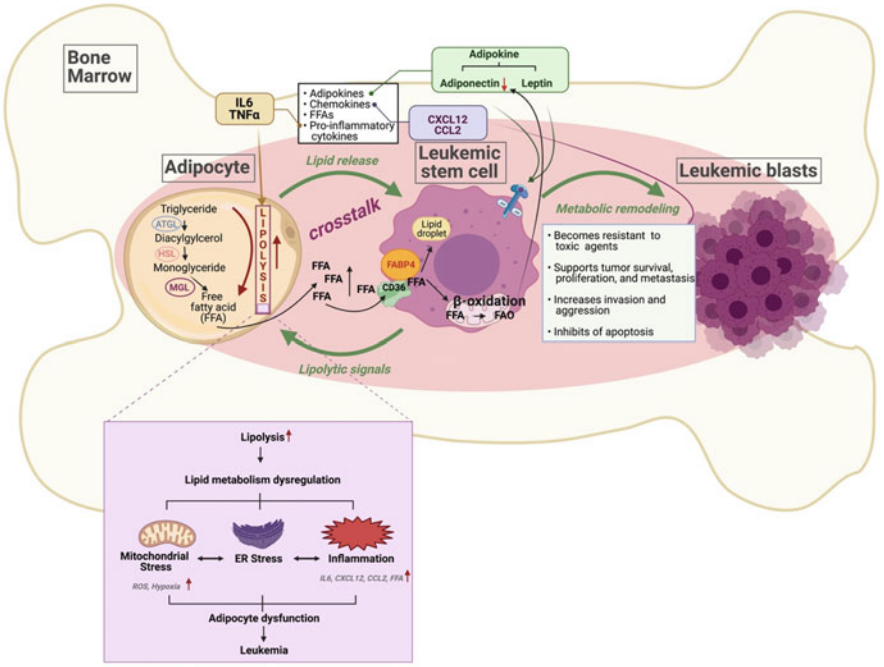

Fig. 3 Summary of the close interactions between the marrow adipose tissue and leukemic cells. This results in metabolic remodeling in the leukemic cells leading to leukemic blasts (Created with BioRender.com).

Horowitz et al. 2017). Due to the heterogeneity in isolation methods and lack of standardization, it is difficult to establish characteristics of the MAT.

The BM comprises the red and yellow marrow, the latter representing the fatty marrow in the long bones that increases with age and makes up 70% of the mass within all BM cavities in adulthood. The remaining hematopoietically active red marrow is highly vascularized (Scheller et al. 2015; Boroumand et al. 2020). Two subpopulations of MAT have been described: constitutive (cMAT) or the regulated marrow adipose tissue (rMAT). Distal marrow sites are filled with cMAT. It is a homogenous and stable marrow space in which adipocytes interact with other adipocytes, are larger in size, do not respond to environmental stressors, are relatively resistant to lipid loss and remodeling, and store monounsaturated fatty acids, i.e., myristoleic and palmitoleic acids (Sahebekhtiari and Tavassoli 1976).

On the other hand, rMAT is present in the proximal, central, and endosteal skeletal sites that develop postnatally, are readily altered by environmental stimuli, maintain multicellular contact, and accumulate saturated fatty acids, i.e., myristic acid and palmitic acid. These adipocytes are smaller in size and interspersed among hematopoietic and other cell lineages in the marrow. They interact with various cell types (Craft et al. 2018) and affect cell fates. rMAT adipocytes are remodeled in response to endogenous and exogenous stimuli, and may show expansion in obesity

(Scheller et al. 2015). It is suggested that (Horowitz et al. 2017) cMAT and rMAT represent different populations of MAT arising from diverse population of progenitors.

BM adipocytes are morphologically similar to white adipocytes as they contain a single fat globule instead of multilocular, mitochondria-rich brown adipocytes. They also express brown adipocyte markers (uncoupling protein 1-UCP1), peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α-PGC-1α, PR domain-containing protein 16-Prdm16, Forkhead box protein C2 (FoxC2), and β3-adrenergic receptor at low levels, and may show characteristics of beige adipocytes depending on the microenvironment needs. Another unique metabolic feature of MAT is the cholesterol-oriented metabolism (Attané et al. 2020) and altered lipolysis associated with a profound downregulation in the expression of monoacylglycerol lipase leading to monoacylglycerol accumulation. Although BM adipocytes show enrichment in proteins involved in cholesterol metabolism correlating with increased free cholesterol content, proteins involved in lipolysis were downregulated in both basal and induced conditions. It was shown that in obesity, through activation of PPARγ, rMAT undergoes hyperplasia, hypertrophy, and whitening (Scheller et al. 2019). Considering the crosstalk between adipocytes and marrow cells, it may be suggested that whitening of rMAT may affect hematopoiesis.

There are conflicting reports in the literature about the role of BM adipocytes on hematopoiesis. The association of increased adiposity in patients with BM failure and experimental studies point out the negative role of MAT (Naveiras et al. 2009). In contrast, other studies suggest a hematopoiesis-supportive role (Mattiucci et al. 2018). It was shown that MAT could stimulate MSCs through LepR to skew their differentiation in favor of adipogenesis through a positive feedback mechanism, thus may affect hematopoiesis (Yue et al. 2016). These results demonstrate the highly active interaction between adipocytes and BM cellular elements in healthy and diseased states.

As demonstrated in solid tumors, MAT also plays a role in cancer pathophysiology and is involved in BM metastasis of solid tumors (Liu et al. 2020a). It was shown that especially red marrow is a common site of metastasis. Excessive blood flow in red marrow, adipocyte synthesis of adipokines, chemoattractants, adhesion molecules, angiogenesis, and immune modulation contribute to establishing a pre-metastatic niche and facilitating BM metastasis (Paolillo and Schinelli 2019). The mechanisms involved include homing through E-selectin, SDF1/CXCR4 axis, activation of the PI3K/PTEN/AKT/mTOR signaling pathway, and establishing a microenvironment appropriate for dormant cancer cells. Micrometastases reside in specific BM niches that regulate their transit to and from the bone. These data suggest that MAT plays an important role in BM micrometastases of solid tumors, and the BM microenvironment can maintain tumor dormancy for extended periods (Price et al. 2016).

Studies demonstrating the role of adipocytes favoring the survival of neighboring cancer cells and the contribution of MAT in BM metastasis of solid tumors (Dirat et al. 2011; Price et al. 2016) pointed out a critical role for MAT in hematological malignancies, which is being enlightened in recent investigations. As observed in

solid tumors, free fatty acid supply to leukemic cells has aroused as a relevant mechanism providing support for cancer cell survival. It was shown that BM adipocytes were programmed by AML blasts towards the establishment of a protumoral microenvironment directing intracellular adipocyte metabolism into a lipolytic state, resulting in the release of fatty acids into the microenvironment to provide metabolic support to leukemic cells. Leukemic blasts program BM adipocytes to generate a protumoral microenvironment, and FABP4 is implicated as a fatty acid transporter protein providing lipolysis products to leukemic blasts (Shafat et al. 2017). It was shown that AML blasts induced phosphorylation of hormone-sensitive lipase to activate lipolysis and transfer of fatty acids from adipocytes to AML blasts. In co-culture of adipocytes and AML, FABP4 messenger RNA was upregulated in both; β-oxidation of AML blasts was activated, and inhibition of FABP4 prevented AML proliferation on adipocytes. In vivo experiments further showed increased survival upon knockdown of FABP4 and carnitine palmitoyltransferase, which is essential for ATP production from FA oxidation.

A recent study has shown that BM-MSCs of patients with AML patients demonstrate adipogenic differentiation propensity with implications for leukemia cell support (Azadniv et al. 2020). Gene ontology and pathway analysis revealed adipogenesis to be among the set of altered biological pathways dysregulated in AML-MSCs in which SOX9 expression was decreased. Their experiments showed that increasing the expression of SOX9 reduced the adipogenic potential of AML-MSCs and decreased their ability to support AML progenitor cells. Moreover, other studies demonstrated exosome-mediated remodeling of the leukemic niche and induction of lipolysis to support leukemic growth (Kumar et al. 2020).

These studies have described significant alterations and remodeling of MAT upon exposure to malignant cells in an interactive microenvironment, pointing out cancer cell-driven alterations in the microenvironment and adipocytes. There is a need for further studies investigating the mechanisms of adipocyte-driven leukemia. In that regard, exposure of adipocytes to environmental stressors emerges as an interesting topic that may lead to identifying mechanisms suggesting a primary/critical role in cancer initiation.

Environmental Stressors and Adipose Tissue

Toxic lipophilic substances are widespread in the environment. Many are resistant to degradation and persist in the environment and living organisms for long periods, particularly in adipose tissue due to the lipophilic nature of these compounds and many of their metabolites. Other organs also retain some of the materials, but the primary storage site for the most lipophilic substances is adipose tissue. There are studies in the literature about environmental toxic agents and adipocytes, but not specifically on BM adipocytes. Adipose tissue is an important protective organ against environmental agents, such as persistent organic pollutants (POPs). Typical examples of POPs include chlorinated compounds such as organochlorine pesticides, polychlorinated biphenyls, and dioxins. The primary source of external

exposure to these chemicals is POP-contaminated food, especially fatty animal products such as fish, meat, and milk. It has been shown that once POPs enter the body, they are distributed through the lymph and blood to their primary deposition site, which is the adipocyte lipid droplets in adipose tissue. It is stated that compared with other critical organs, being a natural location for lipid storage, adipose tissue is a relatively safe organ for POP accumulation, decreasing the burden on other vital organs before elimination over several years (Lee et al. 2017). In addition to strong lipophilic POPs with long half-lives, less lipophilic chemicals with brief half-lives, such as polycyclic aromatic hydrocarbons (PAH), were also detected in adipose tissue. Therefore, adipose tissue is suggested as an organ storing various exogenous chemicals that are not easily metabolized and excreted from the body. In an experimental study, redistribution of hexachlorobenzene from adipose tissue to critical organs such as the brain and kidneys was reported upon weight loss, reversed after weight gain. These results provide evidence for the storage role of adipocytes for environmental toxic agents (Jandacek et al. 2005).

However, the effects of this storage on adipocytes on cancer initiation are not clearly defined, both in the peripheral adipose tissue or MAT. It may be speculated that POP accumulation in adipocytes, even at low doses, upon chronic exposure may lead to establishing a dysregulated microenvironment, adipocyte inflammation, and play a cancer-initiating role. Epidemiological and experimental evidence has linked low-dose POP exposure to obesity-related metabolic dysfunctions such as type 2 diabetes and metabolic syndrome, suggesting dysregulation of adipocyte metabolism (Lee et al. 2014). However, there is a lack of data about POP accumulation in MAT and its possible effects on hematopoiesis and the development of leukemia. This issue may have clinical implications, particularly in diseases with increased susceptibility to mutagenic agents such as Fanconi anemia.

Conclusions

Upon realizing the critical roles of the microenvironment and stem cell niches in maintaining homeostasis and the pathophysiology of diseases, many investigations have suggested tumor microenvironment as a novel target to treat malignancies. Being the main reservoir of stem and progenitor cells, and given its critical role in regeneration, the BM is frequently affected in pathological conditions. Most studies on MSCs were designed to elucidate the changes in phenotypic, molecular, secretory, functional characteristics in different diseases, including hematological malignancy. Leukemic cell-driven alterations in MSCs were shown to contribute to leukemic progression. There is a lack of information for a leukemia-driver role for the BM microenvironment except for several recent reports describing the development of leukemia in experimental models, in which the induced defects in the MSCs and other microenvironmental components led to a dysregulated environment and malignant transformation.

It has been shown that BM adipocytes, upon interaction with leukemic blasts, undergo alterations and provide free fatty acids to leukemic cells for their survival. However, MAT-driven leukemia is not described. Considering the role of adipose

tissue as a depot for environmental pollutants, it may be speculated to have implications as a leukemia driver through the acquisition of epigenetic alterations upon chronic exposure. There is a need for studies focusing on BM adipocytes and leukemia pathogenesis. Fanconi anemia, a disease with DNA repair defect and increased susceptibility to toxic, mutagenic agents, appears to be an appropriate model disease for MAT investigations. Studies in other hematological pathologies and conditions, such as GCSF exposure, will contribute to understanding the role of MAT in disease pathogenesis. However, at first, there is a need for a better description of the MAT and standardization of methods for isolation and characteristics.

Many different methods are being used in in vivo and in vitro experimental studies to identify the BM niche, ranging from studies on cells obtained by different isolation techniques to those using combinatorial approaches with next-generation technologies enabling simultaneous analysis of many BM subpopulations. In light of these studies, there is a need for more studies on human BM samples in relevant diseases and conditions for timely clinical translation of the accumulated scientific knowledge. This may contribute to the identification of therapeutic targets of the BM microenvironment, including the MAT.

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Advances, Opportunities, and Challenges in Stem Cell-Based Therapy

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Renata Szydlak

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Abstract

In recent years, stem cell-based therapy is being widely and intensively investigated. Nowadays modern treatment strategies with mesenchymal stem cells (MSCs) in translational medicine are met with great enthusiasm by scientists and clinicians. The extraordinary properties of MSCs that are better known and understood mean that new possibilities of their application are constantly being tested. Due to their ability to self-regenerate, secrete biologically active molecules and exosomes, differentiate into several cell types, and participate in immunomodulation, MSCs have become a promising tool in the development of modern treatment strategies. The readily available and enormous potential of MSCs allows for a variety of clinical applications in the treatment of many diseases that have hitherto been called "incurable." Most of the results of administering MSCs in clinical trials confirmed the safety and showed promising beneficial

R. Szydlak (✉)
Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland
e-mail: renata.szydlak@doctoral.uj.edu.pl

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results. The therapeutic effects of MSC-based treatments are still not spectacular, and many features of MSCs have not yet been thoroughly investigated, so MSCs continue to be the source of controversial opinion and much debate about these cells. In this chapter, we focus on summarizing the current state of knowledge about the complex nature of MSCs that can be applied to regenerative medicine.

Keywords

iPSCs · Mesenchymal stem cells · MSCs-based therapy · Pluripotent · Stem cells · Transplantation · Regenerative medicine

List of Abbreviations

ALS	Amyotrophic lateral sclerosis
AT-MSCs	Adipose tissue-derived mesenchymal stem cells
BDNF	Brain-derived neurotrophic factor
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CD	Crohn's disease
CFU-F	Colony-forming unit–fibroblast
CMV	Cytomegalovirus
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
GvHD	Graft versus host disease
HLA	Human leukocyte antigens
HO	Heterotopic ossification
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
IPSCs	Induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
MNC	Mononuclear cells
MPCs	Mesenchymal progenitor cells
MSCs	Mesenchymal stem cells
NGF	Nerve growth factor
OA	Osteoarthritis
OI	Osteogenesis imperfecta
SSCs	Somatic stem cells
WJ	Wharton's jelly

Introduction

Cell therapy is a modern therapeutic approach based on cells as therapeutic agents (Gálvez et al. 2011; Ciccocioppo et al. 2021). In regenerative medicine, examining and correctly determining the type of cell to be used in a particular treatment is

essential to the success of therapy. Research to date suggests that stem cells can be used in regenerative medicine due to their unique features of self-renewal as well as differentiation (cell plasticity) into specialized cells with specific functions (Ratajczak and Suszyńska 2013). For this reason, their safety and the ability to repair, replace, or restore the biological function of damaged tissues and organs should be defined (Ciccocioppo et al. 2021). Currently, mesenchymal stem cells (MSCs) constitute the well-characterized and most used cell type in the clinical trials. Despite the low proliferative potential and limited plasticity compared to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), MSCs are easier to obtain from diverse tissues, and their manipulation is free from any ethical moral issues; they have a high in vitro expansion capacity and a low teratogenicity (Sancricca 2010; Trounson and McDonald 2015). All of these properties are in addition to their ability to produce cytokines, growth factors, and microvesicles loaded with bioactive molecules as part of their paracrine activity, migrate and home-in to the site of tissue damage to participate in the repair process, and exert an immunomodulatory effects (Haider and Ashraf 2005). Given these properties, the research and development of MSCs as a drug can help provide new therapeutic alternatives for of high potential in regenerative medicine and cell therapy for diseases that so far do not have effective conventional treatments (Nauta and Fibbe 2007; Gálvez et al. 2013).

In this chapter, the advantages, disadvantages, and side effects of MSC-based therapies have been discussed. In particular, the therapeutic benefits of exogenously delivered MSCs have been discussed, focusing graft versus host disease (GvHD), Crohn's disease, cardiovascular diseases, and orthopedic and neurological disorders.

Mesenchymal Stem Cells

Friedenstein's discovery of the presence of non-hematopoietic stem cells in the bone marrow of animals in the 1970s changed the current outlook on somatic stem cells (SSCs) (Friedenstein et al. 1970; Friedenstein et al. 1976). Friedenstein identified and reported a population of spindle-shaped cells similar to fibroblasts but with colony forming potential. He named these cells as colony-forming unit–fibroblast (CFU-F). Later, these cells were named MSCs or mesenchymal stromal cells (Horwitz et al. 2005). They constitute a heterogeneous group of cells, primitive, and with multilineage potential. The best-known source of MSCs is bone marrow (Wexler et al. 2003; Dominici et al. 2006); however, cells with similar morphology and characteristics can also be isolated from other tissues, including umbilical cord blood, Wharton jelly, placenta, peripheral blood, tissue adipose tissue, and skin (da Silva 2006). In all these organs and tissues, they are housed in specific niches, which are now being studied by high-throughput screening (Ghaemi et al. 2013). They are an integral part of the hematopoietic stem cells (HSCs) niche, where they offer both physical and chemical support to HSCs by secreting bioactive molecules (Crippa and Bernardo 2018).

During in vitro culture, MSCs adhere to the plastic substrate, show high proliferative potential, and the ability to differentiate into cells of mesodermal origin, especially adipocytes, chondrocytes, and osteoblasts. To overcome the controversy regarding the functional potential of MSCs as well as the nomenclature, in 2006, the International Society for Cellular Therapy (ISCT) attempted to define the essential / basic criteria for their identification. In the resulting classification, MSCs must, in addition to adherence to the substrate and trilineage differentiation potential, express surface antigens considered specific for this population, i.e., CD105 (endoglin), CD73, CD90, but lack the expression of hematopoietic cell-specific antigens, i.e., CD34, CD45, CD14 besides the absence of CD11b, CD79a, CD19, and HLA class II antigens expression (Dominici et al. 2006). As our knowledge progresses, and with emerging controversy regarding their functionality, some researchers have started to believe that the criteria set by ISCT based on current literature data should be revisited (Lv et al. 2014). The high similarity of MSCs with fibroblasts in appearance and the lack of harmony in the expression of surface markers specific only for MSCs has led to doubts and difficulties in identifying "true MSCs" (Halfon et al. 2011; Kundrotas 2012; Lupatov et al. 2015). Hence, some researchers suggest that the minimum criteria proposed by ISCT for identifying MSCs are insufficient because MSCs isolated from various tissues represent a relatively heterogeneous population of cells for the expression of surface markers, the ability to proliferate and differentiate (Hass et al. 2011; Pevsner-Fischer et al. 2011; Maleki et al. 2014). Therefore, the ISCT paper issued in 2019 recommends using the acronym "MSC" but supplemented with the tissue source origin of cells, which would highlight tissue-specific properties of the cells being used (Viswanathan et al. 2019). These suggested criteria will help in interpreting the data and the difference in the properties of the cells from diverging tissue sources.

As described earlier, MSCs constitute a heterogeneous population of cells that differ in their proliferative potential and differentiation capacity depending on their tissue location. Bone marrow-derived MSCs (BM-MSCs) can differentiate more efficiently into bone and cartilage as compared to their counterparts derived from the adipose tissue (AT-MSCs) (Im Il et al. 2005; Afizah et al. 2007; Pevsner-Fischer et al. 2011). Similarly, a head-to-head comparison revealed that BM-MSCs were superior in chondrogenic potential than umbilical cord blood-derived MSCs (Contentin et al. 2020). Hence, it is now generally believed that for applications in regenerative medicine, MSCs should be sourced depending upon the treatment outcome. It has now also been shown that in the MSCs population, only a fraction of cells meet the "parental" criteria, while the remaining cells may fulfill a helper function or are the cells capable of differentiation only in one direction (Siegel et al. 2013). Besides, MSCs may also contain a pool of much less advanced and immature cells that express pluripotent transcription factors such as Oct-4, Sox2, and Nanog, and are similar to ESCs (Kuroda et al. 2010; Ogura et al. 2014; Musiał-Wysocka et al. 2019). Reports on the pluripotent properties of the MSCs have not been fully explained. Similarly, a population of small juvenile cells is present in the bone marrow stromal cells with therapeutic potential (Okada et al. 2011).

Mesenchymal Stem Cells Sources

The data published during the last decade have revealed the possibility of obtaining a variety of stem cells from fetal and adult tissue sources, respectively grouped as fetal and adult stem cells, also known as somatic stem cells. The best known and identified source of stem cells by far is the bone marrow. It is inhabited HSCs fraction and MSCs. For 105 mononuclear cells total, it has been shown that there are approximately 1–4 MSCs (Pittenger et al. 1999). In one of the published studies, flow cytometric analysis of BM cells revealed as little as 0.0017 to 0.02% CD271+ CD45- MSCs which adhered to plastic surface and could undergo trilineage difference when challenged with appropriate cues (Alvarez-Viejo et al. 2013). The highest number of MSCs are found in the bone marrow of newborns, which significantly declines with age (D'Ippolito et al. 1999; Stolzing et al. 2008).

BM harvesting protocols are invasive and inconvenient for the donor, and require anesthesia. Therefore, alternative sources of MSCs are being explored for use in cell-based therapy in regenerative medicine. In recent years, adipose tissue is a fairly popular source of MSCs (AT-MSCs). The prevalence of MSCs in adipose tissue is much greater than in the bone marrow, and 1 g of adipose tissue contains 500 times more MSCs than 1 g of BM (Fraser et al. 2006; Kitagawa et al. 2006). Adipose tissue-MSCs have a high proliferation rate and multilineage differentiation capacity within the mesodermal germ layer derivative cells, which renders them a favorable source for cell-based therapy from the clinical perspective. However, characterization of AT-MSCs reveals that the proliferation and differentiation potential of AT-MSCs was significantly influenced by the cell donor, his age, and BMI (Yang et al. 2014). Based on recent reports, the frequency of MSCs and limitations resulting from the amount of material available for collection, adipose tissue is considered a better source of therapeutic cells than BM (Fujimura et al. 2009).

Tissues remaining after delivery, the postpartum tissues (umbilical cord blood, umbilical cord, placenta, membranes, and amniotic fluid) provide excellent primitive cells source. Obtaining tissue material from these sources for stem cell isolation is simple and does not require complex surgical procedures. Compared to MSCs derived from adult BM or AT, MSCs isolated from perinatal tissues are more primitive (Moretti et al. 2010; Lindenmair et al. 2012). Studies have shown that MSCs from postpartum tissues can differentiate into cells from all three germ layers, which indicates their greater differentiation potential, and therefore pluripotency and primitiveness (Guillot et al. 2007). However, obtaining cells from postpartum tissues is a time-constrained exercise and requires immediate isolation of cells or freezing tissues in biobanks. The procedure of obtaining MSCs from frozen UCB or umbilical cord deposits is still ineffective due to the lack of an optical protocol. Moreover, it is believed that UCB is a relatively unfavorable source for obtaining MSCs, in contrast to the rich fraction of other mononuclear cells (MNCs). Wharton's jelly (WJ) – umbilical cord tissue is also a valuable source of MSCs. The WJ-MSCs collection procedure is technically simpler and a rich population of post-fetal MSCs can be efficiently isolated (Batsali et al. 2013; Nagamura-Inoue 2014). The

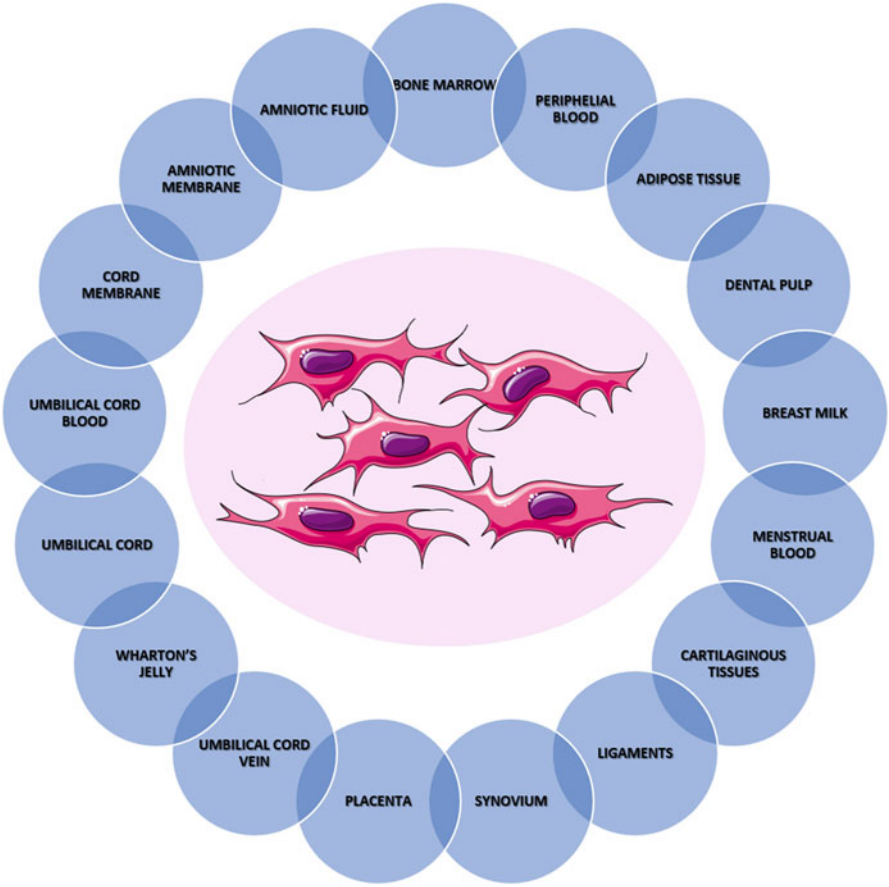

Fig. 1 Summary of the possible tissue sources for mesenchymal stem cells

published data show that Wharton's jelly contains much higher propensity of MSCs compared to the UCB (Zeddou et al. 2010; Pelosi et al. 2012). The possible tissue sources of MSCs have been summarized in Fig. 1.

The Therapeutic Potential of Mesenchymal Stem Cells

Despite numerous in vitro and in vivo studies, as well as therapeutic benefits confirmed in clinical trials, the exact mechanism of therapeutic benefits of MSCs is still not fully understood. Based on the results obtained in preclinical and clinical studies, it can be concluded that MSCs certainly has a unique ability to immunomodulate, regenerate, and heal the damaged tissues. The available experimental data make it possible to dissect and understand some of the mechanisms responsible for the therapeutic potential of MSCs. The therapeutic efficacy of

exogenously injected MSCs may result from both cell-cell interactions and the secretion of biologically active molecules. Hence, the therapeutic effect can be obtained exploiting the three main properties of MSCs: differentiation into several types of tissues, the immunomodulatory effect, and the ability to influence the intrinsic repair process through the paracrine secretion of appropriate cytokines and direct contact with other cells. MSCs act as a local coordinator of tissue repair in most cases. Their advantage over the use of other mediators (cytokines) is the cross talk between MSCs and other cells and tissue regeneration mediators. This close interaction MSCs with their local environment enables them to adapt well to the changing situation, e.g., inhibition of the inflammatory reaction in the first phase of regeneration and the production of stimulators for cell proliferation and differentiation in the next phase.

Application of Mesenchymal Stem Cells in Medicine

The number of clinical trials using MSCs in regenerative medicine (ClinicalTrials.gov) is growing rapidly. There are currently ten approved MSCs-based therapies available for a variety of disorders in the pharmaceutical market worldwide (Table 1).

The experimental in vitro data and the promising results from preclinical studies and translational studies show that cell-based therapy with MSCs therapeutic benefits for patients with various diseases. However, despite receiving encouraging data from these studies over the past decade or more, many questions related to the biology of MSCs and hence, their usefulness as choice cells remain open for discussion and further investigation. For example, there remain some uncertainties between the immunophenotype of MSCs with relevance to its functionality and the procedural hiccups, including posttransplant survival, route of administration, and type of transplant (autologous or allogeneic). Moreover, it also remains less well-explored about the properties of cells, such as the potential for in vitro trans-differentiation, persist after transplantation.

MSC-Based Therapies in Graft Versus Host Disease

Graft versus host disease (GvHD) accompanies allogeneic hematopoietic stem cell transplantation (HSCT) in many patients. Corticosteroids are used to treat GvHD; however, this therapy is ineffective in all patients (Martin et al. 2012). The immunomodulatory properties of MSCs described in experimental studies suggest their use in the treatment of GvHD (Mohanty et al. 2020). Many studies have proven that MSCs can modulate the function of the immune system and have found their use as companion cells in transplantation in the treatment of GvHD (Weng et al. 2010; Zhao et al. 2015). Indeed, MSCs transplantation and their derived exosomes have recently been performed to prevent or treat GvHD, especially in patients who do not respond to steroids (Le Blanc and Mougiakakos 2012; Elgaz et al. 2019;

Table 1 MSCs-derived products with regulatory approval

MSC product (Company)	MSC tissue-source	Indication	Approval granted (year)
Queencell (Anterogen Co. Ltd.)	Autologous Human AT-MSCs	Subcutaneous tissue defects	South Korea (2010)
Cellgram-AMI (Pharmicell Co. Ltd.)	Autologous Human BM-MSC	Acute myocardial infarction	South Korea (2011)
Cupistem (Anterogen Co. Ltd.)	Autologous Human BM-MSC	Crohn's fistula	South Korea (2012)
Cartistem (Medipost Co. Ltd.)	Allogeneic Human UC-MSC	Knee articular cartilage defects	South Korea (2012)
Prochymal, remestemcel-L (OsirisTherapeutics Inc., Mesoblast Ltd.)	Allogeneic Human BM-MSC	GvHD	New Zealand (2012) Canada (2012)
Neuronata-R (Corestem Inc.)	Autologous Human BM-MSC	Amyotrophic lateral sclerosis	South Korea (2014)
Temcell HS (JCR Pharmaceuticals)	Allogeneic Human BM-MSC	GvHD	Japan (2015)
Stempeucel (Stempeutics Research PVT)	Allogeneic Human BM-MSC	Critical limb ischemia	India (2016)
Alofisel (TiGenix NV/Takeda)	Allogeneic Human AT-MSC	Complex perianal fistulas in Crohn's disease	Europe (2018)
Stemirac (Nipro Corp)	Autologous Human BM-MSC	Spinal cord injury	Japan (2018)

Zhang et al. 2017). However, despite the use of MSCs in many clinical trials, there is still controversy about the benefits of such therapy. Although a reduction in inflammatory processes is observed after MSCs implantation, a reduction in the immune response may increase the risk of infection, especially in patients receiving immunosuppressive therapy after HSCT (Nauta and Fibbe 2007). It has been reported that infusion of MSCs may dangerously limit the antimicrobial immune response (Balan et al. 2014). A clinical trial published by Ning et al. showed that the incidence of acute and chronic GvHD in MSCs-transplanted recipients was lower than in the non-MSCs transplanted patients, but the episodes of severe infections were more significant in patients who received bone marrow-derived HSCT and MSCs than in the control group who did not receive the MSCs treatment. Among patients, two developed CMV interstitial pneumonia and/or fungal infection (Ning et al. 2008).

Forslöw et al. suggest an increased susceptibility to pneumonia observed in patients with GvHD after MSCs infusion (Forslöw et al. 2012). High-peak CMV viral load was found in a retrospective study of patients with steroid-resistant GvHD receiving MSCs (von Bahr et al. 2012a, b). This contradicts the previous in vitro experiments, which showed that anti-CMV cytotoxic T cells were limited to the BM-MSCs effect (Karlsson et al. 2008). Recently, Thanunchai et al. postulated that in viral infections, human BM-MSCs might also act as viral transmitters (von Bahr et al. 2012a, b). There is a suggestion that MSCs may lose their immunosuppressive properties in mismatched settings, which has been shown in murine cells (Badillo et al. 2008). Moreover, the study by Muroi et al. showed that the transplanted BM-MSCs in the acute phase II/III GvHD study did not protect the development of chronic GvHD (Muroi et al. 2016).

Based on the above studies, it should be emphasized that MSCs transplantation for the prevention or treatment of GvHD is relatively safe and effective in steroid-resistant GvHD, but infections remain a major risk for patients. Moreover, it has been shown that MSCs transplanted due to established GvHD may cause increased relapse (Ning et al. 2008). In a recent study by Ringden et al., the authors mentioned several side effects following transplantation of residual placenta-derived MSCs in the treatment of GvHD. Among them, relapse, pneumonia, bacterial, viral, and fungal infection, and transplant failures have been listed (Ringden et al. 2018). A new strategy to support a high frequency of MSCs effects on GvHD with a little adverse effect on the patient appears to be warranted in large-scale randomized trials. Research by various laboratories focuses on developing new MSCs-based drugs. One of the first MSCs-based drugs approved for the treatment of GvHD was Prochymal.

Mesenchymal Stem Cell-Based Therapies in Crohn's Disease

Crohn's disease (CD) is classified as a chronic inflammatory disease that mainly affects the gastrointestinal tract. In CD, the formation of an anal fistula is difficult to treat and is associated with a large number of complications, including the risk of bowel resection. Moreover, the perianal fistulas arising in the course of CD are difficult to treat with standard drugs and surgical procedures (Veauthier and Hornecker 2018). Despite significant advances in the techniques used, treatment of patients with CD remains a difficult task with a high risk of relapse (Gisbert et al. 2015). Recently, satisfactory therapeutic benefits have been obtained by administering MSCs. However, the indications for the use of MSC-based therapy in CD concern mainly perianal fistulas (Zhang et al. 2017).

Based on the available results from preclinical and clinical studies, it can be assumed that the therapeutic mechanism of MSCs in this disease is mainly based on the immunomodulatory effect. MSCs have been shown to inhibit T cell proliferation mediated by indoleamine 2,3-dioxygenase (Wang et al. 2018). Thanks to their enormous regenerative potential, MSCs are currently used in the treatment of fistulas in Crohn's disease and other etiologies. Data collected from the analysis of various

clinical cases show that complete healing of the fistula can be achieved after several local administrations of MSCs either alone or in combination with infliximab and azathioprine (Forbes et al. 2014; Moniuszko et al. 2018). The first EMA-approved MSC-based drug for the treatment of complex perianal fistulas in CD was Alofisel. Recent advancement in this regard is the use of acellular products of MSCs based on the use of extracellular vesicles derived from MSCs. A direct comparison of cell-based and cell-free approaches based on MSCs and their derivative extracellular vesicles has been provided by Li et al. in an experimental mice model of DSS-induced colitis (Li et al. 2020) and reviewed by Ocansey et al. (2020).

Mesenchymal Stem Cell-Based Therapies in Cardiology

Cardiovascular diseases are one of the most common causes of death worldwide (Virani et al. 2020). The currently used method of treating a "fresh" heart attack consists of administering a thrombus-dissolving drug as soon as possible and performing a cardiological intervention in the form of opening the lumen of a closed arterial vessel, which is aimed at limiting damage and then necrosis of the heart tissues (Peng et al. 2016).

As the heart exhibits limited endogenous regenerative capacity, although the long-standing dogma about the heart has been challenged due to the presence of resident cardiac stem cells (Takamiya et al. 2011; Lu et al. 2013; Belostotskaya et al. 2015), cell-based therapeutic approach is currently the subject of much preclinical as well as clinical research. However, a significant focus of cell-based therapy is on MSCs as choice cells, due to their superior biological and functional characteristics, to demonstrate the effectiveness of using MSCs, either naïve or preconditioned or genetically modified (Changfa et al. 2017), to reduce postinfarction scars and restore normal contractile function in the infarcted heart (Haider et al. 2008; Kim et al. 2009; Haider et al. 2010; Ahmed et al. 2010; Afzal et al. 2010; Suzuki et al. 2010). Despite encouraging data, however, the exact mechanism by which MSCs contribute to myocardial regeneration is still not fully understood (Lpez et al. 2013; Zhao et al. 2015). Although MSCs show great potential for pro-chondrogenic, osteogenic, and adipogenic differentiation, several studies have provided evidence that under optimal in vitro culture conditions or in the cardiac microenvironment in vivo post engraftment, MSCs can also give rise to other highly specialized tissue types, including cardiomyocytes and endothelial cells (Shim et al. 2004; Aguilera et al. 2014; Haider et al. 2008) and lead to stable therapeutic benefits (Jiang et al. 2008). Given these findings, MSCs have been extensively tested as a source of cells to replace damaged myocardial tissue in vivo, in both acute and chronic cardiac injury, confirming the ability to transdifferentiate MSCs into cardiac and endothelial cells (Haider 2006; Dawn et al. 2009). However, the ability of MSCs to differentiate into functional endothelial and cardiac cells in vivo has not been fully established. Moreover, it is difficult to confirm the transplanted integrated cells in any tissue in vivo due to the lack of specific MSCs markers (Lin et al. 2013). However, the increase in recent evidence from many laboratories strongly indicates the

overwhelming paracrine effect in MSCs after heart transplant to promote cell survival, proliferation, and differentiation by MSCs resulting from secreted bioactive factors and extracellular vesicles (EVs) (Nazari-Shafti et al. 2020; Lei and Haider 2017). Thus, these two main mechanisms, including (1) direct differentiation of MSCs into cardiac and endothelial cells and (2) paracrine activity mediated by soluble molecules and MSC-derived vesicles, are now considered to be the primary mediators of the beneficial effects of MSC-based therapies (Majka et al. 2017; Szydlak 2019; Nazari-Shafti et al. 2020). Most of the beneficial effects after MSCs injection are believed to be related to their paracrine effects on endogenous cells, resulting in increased vasculogenesis and angiogenesis, as well as increased cell survival (Haider and Aziz 2017). The role of the paracrine activity of MSCs is considered so dominating that cell-free therapy approach using MSCs conditioned medium rich in soluble factors (i.e., growth factors, cytokines) and insoluble factors (i.e., exosomes) is emerging as an alternative to cell-based therapy (Haider and Aramini 2020; Haider and Aslam 2018). Given their robust nature, MSCs have also been reprogrammed to iPSCs to achieve a continuous source of cardiac progenitor cells for use in cardiac repair in an experimental animal model (Buccini et al. 2012).

Although MSCs, from adult tissue sources, represent one of the safest stem cell populations, with almost no risk of the endogenous teratogenic potential of normal pluripotent stem cells such as ESCs and iPSCs, in vivo application of MSCs to heart tissues could still potentially lead to some undesired effects post engraftment (Price et al. 2006; Breitbach et al. 2007). The few reported safety concerns for MSCs are related to their possible (1) pro-arrhythmic and (2) carcinogenic capacity in heart tissue, as well as (3) differentiation into undesirable tissue types (Price et al. 2006; Breitbach et al. 2007). Price et al. report that BM-MSCs administered intravenously to pigs with acute ischemia/reperfusion injury improves cardiac parameters and reduces adverse wall thickening but may also adversely affect the electrophysiological properties of the myocardium, suggesting the pro-arrhythmic potential of these cells. However, the beneficial effects of injected MSCs on cardiac function and anatomy observed in this study were greater than the recorded arrhythmic events, and ultimately the authors concluded the efficacy of MSCs in the heart repair model, however, with a note of caution (Price et al. 2006). On the other hand, numerous clinical studies in patients suffering from acute or chronic ischemic heart disease have shown very little or no adverse effect of MSCs on the electrical properties of the myocardium after transplantation, as summarized in several reviews and meta-analysis reports (Afzal et al. 2015). Consequently, reported events of pro-arrhythmic MSCs activity are somewhat rare and pharmacologically treatable; however, this should be considered and assessed, especially during clinical trials, as a potential risk identified in some animal studies (Menasché 2009). Some of the unresolved problems in clinical trials in cardiology, besides the source and quality of the cell preparation used during the trials (Shahid et al. 2016; Haider 2018), may also be partly related to an insufficient number of placebo groups. However, compared to other stem and progenitor cells used to repair the heart, including skeletal muscle myoblasts, widely studied in the early 2000s, MSCs can be considered cells with a limited risk of arrhythmia in the heart tissue (Haider et al. 2004; Kahn 2006).

In summary, the positive therapeutic results of exogenously injected MSCs are probably due to several mechanisms of their action, namely the ability of MSCs to differentiate into cardiomyocytes, smooth muscle cells, vascular endothelial cells, and the ability of MSCs to secrete multiple cytokines and trophic factors including the insoluble factors, i.e., exosomes (Majka et al. 2017; Szydlak 2019). Due to their immunosuppressive properties, MSCs may also help to alleviate inflammation and stimulate endogenous repair mechanisms.

Mesenchymal Stem Cells-Based Therapies in Orthopedics

Previous studies have shown a beneficial effect of MSCs primarily in the treatment of the osteoarticular system. Numerous studies with various animal models of orthopedic disease have documented the multipotential properties of MSCs, showing their ability to differentiate in multiple tissues such as muscle, bone, cartilage, and tendons (Kingery et al. 2019). Their use as an adjunct to orthopedic surgery is also being explored to ensure rapid wound healing (Murrell et al. 2015). However, contrary to the initial assumption that the therapeutic benefits of MSCs depend on their cell replacement capacity via transdifferentiation, recent studies have shown that the paracrine function of MSCs remains the primary mechanism by which they participate in the tissue repair post engraftment (Von Bahr et al. 2012a, b). MSCs have been reported to exhibit immunosuppressive and immunomodulatory properties by secreting specific factors that may modulate inflammatory responses following orthopedic trauma (Marcucio et al. 2015). However, the common mechanism of action of MSCs in orthopedic applications has not been fully established (Berebichez-Fridman et al. 2017).

Many reports have summarized the role of MSCs in the treatment of osteoarthritis (OA). Several experiments in animal models of knee OA have shown that MSCs-based therapy may delay progressive degeneration of the joint (Shimomura et al. 2018; White et al. 2018). Most human studies support the notion that short-term use of MSCs is safe and feasible; however, further experimentation is necessitated. Importantly, we still need clear evidence to support the effectiveness of MSCs transplantation in OA patients (Jihwan et al. 2021). In randomized controlled clinical trials, injection of MSCs used to treat knee OA is effective (Lamo-Espinosa et al. 2016; Park et al. 2016).

However, the results reported by Shim et al. and Pas et al. disclosed that after MSCs injections for the treatment of knee OA, only a few cells survived at the injection site (Shim et al. 2015; Pas et al. 2017). Moreover, the optimal therapeutic dose of cells, co-adjuvants, and uptake source has not yet been optimized (Lamo-Espinosa et al. 2016). The use of MSCs in cartilage repair has a significant placebo-related limitation because the tissue sampling procedure makes it difficult to perform a blind design study (Filardo et al. 2016). Therefore, new studies using MSCs for orthopedic patients must be performed with greater care and under controlled ex vivo preparation conditions to assess their therapeutic efficacy in these patients ultimately.

Currently, there are attempts to research the use of MSCs in osteogenesis imperfecta (OI), a genetically determined disease associated with the production of an abnormal form of type I collagen (Horwitz et al. 2002). It has been shown that MSCs transplantation has a beneficial effect on the reduction of skeletal damage. In a clinical trial, prenatal transplantation of MSCs in 31-week-old fetuses with ultrasound confirmed diagnosis of osteogenesis imperfecta showed significant improvement in patients' condition. MSCs transplantation in patients lowered the incidence of fractures and skeletal abnormalities (Götherström et al. 2014). Two patients underwent adjuvant transplantation to enhance the therapeutic effect, and at the age of 18 months after birth and 8 years of age, MSCs were re-transplanted. The effects of prenatal transplants in combination with postnatal transplantation resulted in clinical benefits. However, studies of a larger group of patients are needed to evaluate the effectiveness of the proposed therapy fully.

Numerous studies suggest that apart from the potential effect of MSCs on tissue regeneration, these cells may also be significantly involved in the process of heterotopic ossification (HO), i.e., ectopic bone formation in tissues other than bone (Kan et al. 2017). Besides, stem cell-based therapies in orthopedic trauma have identified MSCs contributing to the high osteogenic differentiation (Agarwal et al. 2016). Also, the inflammatory response may stimulate the differentiation of mesenchymal progenitor cells (MPCs) into osteoblasts and osteoblast-like cells. If this process is localized in muscles or other soft tissues, it may directly contribute to the formation of HO (Winkler et al. 2015). It has also been reported that MSCs may be responsible for the recurrence of HO (after surgical resection). In turn, excision of HO may result in the re-emergence of the MSCs population and the signaling mechanisms observed in the original lesion (Agarwal et al. 2017).

MSC-Based Therapies in Neurology

The concept of the clinical application of MSCs seems to be of great hope in the treatment of neurological diseases, both those of a neurodegenerative nature and damage to the central nervous system resulting from stroke or trauma. It seems that MSCs can be used in the direct regeneration of the cellular structure of the nervous system not only because of their immunomodulatory and neurotrophic functions but also due to their potential differentiation abilities and reparability. The neuroprotective and neuro-regenerative properties of MSCs transplantation can be associated with the production of numerous growth, anti-inflammatory, and anti-apoptotic factors important for neurons. The observations of some researchers show that MSCs, due to their abovementioned functions, may be responsible for the protection of newly formed neurons, their proliferation, and maturation. Hence, co-transplantation of neuronal stem cells and MSCs has been used to promote neuronal stem cell survival, proliferation, and differentiation post engraft in experimental animal model of spinal injury (Hosseini et al. 2018).

It has been reported that transplanted human MSCs in an experimental stroke or trauma model in animals can significantly improve motor functions

(Gornicka-Pawlak et al. 2011; Sarnowska et al. 2013). The observed effect was associated with the anti-apoptotic action and production of factors by MSCs that stimulate the survival of neurons (Anbari et al. 2014; Gu et al. 2014; Yin et al. 2014). One of the major factors for successful cellular therapy of stroke is the route of cell delivery to the damaged part of the brain. For this reason, the circulatory system is considered correct. However, it is important to be aware of how the exogenous cells are administered to ensure the patient's safety on the one hand and, on the other hand, to guarantee the maintenance of good quality therapeutic cells during the transplant procedure.

Cui et al. disclosed that cell agglomeration before injection increased in proportion to the duration of the cells kept in suspension (Cui et al. 2016). Moreover, due to their size, MSCs can induce severe vascular occlusions after intravascular delivery. The size of MSCs in an in vitro monolayer culture increases with the number of passages; the solution can create a 3D nodular culture in vitro that will reduce MSCs again (Ge et al. 2014). Failure of a positive result after systemic MSCs administration in stroke was confirmed in another experimental study where the intravenous injection of human BM-MSCs in a mouse model of stroke contributed little to enhancing cell proliferation in neurogenic areas. Moreover, neither a detectable reduction in infarct size nor favorable clinical symptoms were observed (Steiner et al. 2012). Moreover, MSCs delivered intraarterially in a mouse model of ischemia did not improve functional recovery and may further promote the risk of brain damage (Argibay et al. 2017). These multifocal changes contributed to a significant decrease in cerebral blood flow as a result of small vessel obstruction by exogenous cardiovascular cells, while posing a deep risk of secondary embolism in the brain following stroke. A recent advancement in the treatment of stroke is the use of cell-free therapy approach in which MSCs-derived secretome was injected intracerebroventricularly in an experimental animal model of ischemic stroke (Taei et al. 2021).

Another example of a neurological disease in which MSCs-based therapies raise high hopes is amyotrophic lateral sclerosis (ALS). Phase I study results showed no significant positive effects of exogenous MSCs embedded in the spinal cord. The main conclusion from most clinical trials of ALS therapy with MSCs was limited to that of the safety of the treatment. In the study by Syková et al. in which BM-MSCs were transplanted intrathecal, the favorable outcome was seen only in a few patients and was limited to a short time after transplantation (Syková et al. 2017). This outcome may be due to the short survival time of the cells after implantation or the differentiation status of the transplanted MSCs. Repeated administration of cell doses can be crucial to achieving better prognosis; however, this may be challenging in terms of the manufacturing process. Another phase I study with an intrathecal autologous injection of BM-MSCs revealed mild adverse reactions immediately overcoming exogenous cell deposits, such as fever, pain, and headache; however, there were no major treatment effects (Oh et al. 2015). Staff and colleagues performed intrathecal injection of MSCs in the adipose tissue during ALS treatment. The authors did not observe any spectacular improvement in the treated patients and postulated a reduction in enthusiasm for the effectiveness of the therapy (Staffe et al. 2016). Moreover, MSCs transplanted intramuscularly and intrathecal, aspirated from the bone marrow, are

safe and stimulate the release of neurotrophic factors; however, this approach contributed to some disease regression in only half of the patients over the next 6-month period (Petrou et al. 2016). Nevertheless, previous study by Karussis and colleagues in which BM-derived MSCs were injected intravenously and intrathecal did not bring any positive effects in ALS patients (Karussis et al. 2010).

In the treatment of neurological disorders, neurotrophins may play a particularly important role. Neurotrophins play a key role in the differentiation and survival of neurons in the central nervous system and are also involved in synaptic plasticity underpinning learning and memory. The main source of neurotrophic factors are nerve cells, but recently it has been believed that they can also be produced by other cells, including MSCs (Sadan et al. 2012; Paczkowska et al. 2013) (Paczkowska et al. 2013; Sadan et al. 2012). Neurotrophins, in particular brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3, and neurotrophin 4/5, may have a neuroprotective function in multiple sclerosis, slowing the rate of atrophic changes and affecting the functional network of neural connections with improved cognitive functions in the patients. Evidence of the neuroprotective potential of neurotrophins has so far been obtained primarily in the studies conducted in experimental animal models (Lykissas et al. 2007; Gordon 2009). More and more reports suggest that neurotrophins secreted by MSCs may prove useful in many other neurodegenerative diseases: Parkinson's disease, Alzheimer's disease, in the treatment of patients after stroke and spinal cord injuries (Malgieri et al. 2010. Machaliński et al. 2012, Paczkowska et al. 2015).

Potential Risks of MSC-Based Therapies

MSCs-based cell therapy represents a new promising approach in treating many diseases. However, data on the risks and possible long-term side effects of their use still lack despite their positive results. There is also a lack of data, including analyzes of long-term studies in the context of possible threats resulting from the possibility of neoplastic transformation. Although there are no reports to date about teratoma formation after MSCs transplantation, the long-term risk from cell therapy remains less well-studied and underreported in the published data. There are, however, some conflicting reports of spontaneous transformation of MSCs under in vitro culture conditions. Various effects were observed depending on the species used and the source of MSCs the cultivation techniques used, and the in vitro expansion time (Miura et al. 2006; Bernardo et al. 2007; Tang et al. 2013). The risk of spontaneous transformation of MSCs due to prolonged culture has been demonstrated in a study describing the population of cells from the bone marrow and blood (Tang et al. 2013). Bernardo and colleagues have shown that MSCs retained the correct phenotype and morphological structure during in vitro expansion in the optimal culture conditions, besides maintaining normal cell function over a more extended period. During 44 weeks of cultivation, no changes were observed in cell karyotype (Bernardo et al. 2007).

Another problem that arises from MSCs transplantation, apart from direct transformation, may be the risk of stimulation of an already existing neoplastic growth by MSCs. Due to their migratory capacity, transplanted MSCs move to the site of neoplastic growth, stimulate tumor cell proliferation, promote angiogenesis, and support tumor metastasis. In this case, a prior precise diagnosis and selection of patients indicated for cell therapy are necessary. The results showed that the immunosuppressive environment created by MSCs also promoted tumor metastasis in the mechanism of the so-called epithelial-mesenchymal transition (EMT), facilitating cell migration (Ljujic et al. 2013). Studies by other authors have shown that the co-culture of cancer cells with MSCs accelerates tumor expansion (Xu-ting et al. 2009; Zimmerlin et al. 2011). Given the incredible enthusiasm for stem cell-based therapy, care should be taken to consider all the possible adverse effects.

Conclusions

The more recent scientific analysis has shown limited therapeutic effects of treating MSCs, suggesting that the direct regenerative potential of these cells related to their ability to differentiate may not be as effective as previously expected. Several exogenous factors may significantly influence the biological properties of MSCs and ultimately on their therapeutic capacity, optimized protocols for MSCs isolation and ex vivo preparation for clinical use must be well-established and standardized. Such a comprehensive effort should be taken into account by the scientific community focusing on the practical applications of MSCs in tissue repair in terms of the optimal preparation of MSC-based products for more effective patient therapies.

The advantages associated with the use of MSCs in tissue repair, i.e., their safety, relatively broad differentiation capacity, and high paracrine capacity, render these cells an important therapeutic option for further exploration and development as a novel cell-based therapy approach in the future. However, more in-depth and mechanistic research is needed at the preclinical and clinical levels. New research data on MSCs will help determine the effectiveness of cells administered to patients as part of a therapeutic approach. Additional research would also make a significant contribution to the overall biology of stem cells.

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The Stem Cell Continuum Model and Implications in Cancer

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Theo Borgovan, Ari Pelcovitz, Rani Chudasama, Tom Ollila, and Peter Queseneberry

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T. Borgovan (✉) · A. Pelcovitz · R. Chudasama · T. Ollila · P. Queseneberry
Division of Hematology and Oncology, Rhode Island Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, USA
e-mail: theo.borgovan@brown.edu; tb2182@columbia.edu; ari.pelcovits@lifespan.org; rchudasama@lifespan.org; thomas_ollila@brown.edu; pquesenberry@lifespan.org

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Abstract

The hematopoietic stem cell is constantly fluctuating through various intrinsic phenotypic potentials. It has dynamic plasticity and is intimately tied to cell cycle dynamics as well as numerous external stimuli. The salient studies conclude the stem cell exists in a continuum of transcriptional opportunity regulated by multiple variables, including biologically active nanoparticles termed extracellular vesicles (EVs). These bioactive mediators closely impact cellular function and phenotypic potential and play a critical role in the regulation of normal hemostasis, as well as in the development and evolution of various cancers. In this chapter, we explore the integral data explaining the stem cell continuum, the interplay between this model and EVs from various cell types, as well as the role of EVs in various solid and hematologic cancers. Finally, we evaluate the role of EVs as unique and reliable biomarkers across disease states ranging from traumatic brain injury to malignancy.

Keywords

Biomarkers · Cancer · CSCs · Extracellular vesicles · Stem cells · Therapeutics

Abbreviations

ALL	Acute lymphoblastic leukemia
BCR	B-cell receptor
BM	Bone marrow
CAFs	Cancer-associated fibroblasts
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
FIH	Factor inhibiting
GPC1	Glypican-1
HIF	Hypoxia-inducible factor
HUVEC	Human umbilical vein endothelial cells
hWJMSC	Human Wharton's jelly mesenchymal stem cells
IL	Interleukin
LT-HSC	Long-term hematopoietic stem cell
MBC	Metastatic breast cancer
MHC	Major histocompatibility complex
miRNA	MicroRNA
MM	Multiple myeloma
MRP-1	Multidrug resistance protein 1
MSCs	Bone marrow mesenchymal stem cells
MVB	Multivesicular bodies

SDF1	Stromal cell-derived factor 1
TBI	Traumatic brain injury
TD-MSCs	Palatine tonsil-derived MSCs
TF	Tissue factor

Introduction

The Stem Cell Continuum

Bone marrow hematopoietic stem cells (HSCs) have been exhaustively studied. Present dogma is that there is a dormant noncycling lineage negative (lin-) c-kit^{+-} Sca-1^{+}CD150^{+} cell in the marrow, which represents this stem cell (Colvin et al. 2010, 2007). This cell is reported to have extensive differentiation potential, self-renews, and responds to microenvironmental cues. The system is hierarchical, with the stem cells giving rise to a diverse population of progenitor cells, which then differentiate into end hematopoietic cells. Our published data indicate that the bone marrow-derived pluripotent HSCs constitute a population of actively cycling cells which are characterized by continuously changing phenotypes (Colvin et al. 2007). This cell population exists as lin + cells, discarded during conventional stem cell purification (Goldberg et al. 2014). Thus, a cell that is transiting cell cycle is continuously altering its nature. This is consistent with multiple reports of heterogeneity of bone marrow HSCs. Perhaps the most impressive observation is that heterogeneity is observed even with cycle-synchronized stem cells (Colvin et al. 2010). Critical observations have been reported that the long-term repopulating marrow stem cells alter hematopoietic differentiation potential as it transits the cell cycle (Colvin et al. 2007). In these experiments, highly purified lin-rhodamine low Hoechst low murine stem cells showed differentiation predilections as they transit a cytokine-induced cell cycle.

Another critical component of the universal stem cell model is multiple observations of marrow "stem cells" forming non-hematopoietic cells after in vivo engraftment into lethally irradiated mice (Lagasse et al. 2000; Nilsson et al. 1999; Goldberg et al. 2014; Theise et al. 2000; Abedi et al. 2003; Badiavas et al. 2003). Krause and colleagues have demonstrated that single marrow cells can give rise to a wide variety of non-hematopoietic epithelial cells. And more recently, they have identified these cells as a very small embryonic-like stem cell (Krause et al. 2001; Kassmer et al. 2013).

Put together, the observations on cell cycle status of long-term repopulation marrow stem cells, their differentiation potential including their capacity to differentiate into non-hematopoietic end cells, and their intrinsic heterogeneity suggest a new model of stem cell biology. We propose that there are no individual stem cells for different tissues; rather, *a universal stem cell* population exists that functions for most and possibly all tissues in the body. The universal stem cell is a cycling cell and thus is continuously changing phenotype. This includes its differentiation potential that is realized when the universal stem cell resides in the conducive environment. As shown in Fig. 1, the same universal stem cell at one point during the cell cycle in

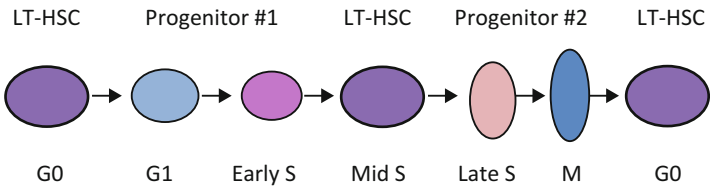

Fig. 1 The cell cycle is a continuum. During hematopoiesis the long-term hematopoietic stem cell (LT-HSC, colored in dark purple) changes phenotype as it transits through cell cycle and fluctuates between different phenotypes (colored in blue, brown, and light purple) and functional potential. (Used with the permission of Chibuikem Nwizu, chibuikem_nwizu@brown.edu)

hepatic tissue will give rise to hepatic cells, while if it is in lung tissue environment, it will give rise to epithelial lung cells. Likewise, it will give rise to hematopoietic cells in the marrow, but the type of end cell differs depending upon cell cycle status; thus, it could differentiate into megakaryocytes, granulocytes, erythrocytes, or other end hematopoietic cells. This model can fit nicely into much of our current data on extracellular vesicle cellular modulation.

Extracellular Vesicle Basics

At first glance, extracellular vesicles (EVs) seem simple, nano-sized, lipid membrane-enclosed particles that are reasonably ubiquitous and are released from essentially all cell types in the mammalian body. Their function unknown at the time, at first discovery they were merely dismissed as cellular waste products, shuttling cellular junk from predominantly red blood cells and platelets. Today, with the recent progress in the field of EVs, researchers have started to appreciate their complexity and robust biological potential. Scientific efforts have only just begun to architect framework taxonomy, unraveling their heterogeneity and pluripotency.

Subsequent work has characterized and subdivided these entities on the basis of size, density, and morphology (Borgovan et al. 2019). Eventually, two basic types of vesicles were defined by differential ultracentrifugation purification: exosomes and microvesicles. Exosomes derived from multivesicular bodies range from 40 to 120 nm in diameter, while microvesicles derived as a result of membrane blebbing range from 50 to 1000 nm in diameter. Other vesicular entities were also defined, including apoptotic bodies as given in Table 1 and Fig. 2.

Extracellular Vesicle Functions

The functional potential harnessed by EVs is immense. They allow for intercellular communication sans direct cell-to-cell contact, honing pertinent information to distinct cells in proximity to the effector cell, or cellular targets located in more distant vicinities.

Table 1 Biological pathways of extracellular vesicles

Vesicle type	Size (nm)	Origin	Content	Marker
Exosomes	40–120	Endocytic pathway	Proteins, lipids, and nucleic acids (mRNA, miRNA, and other noncoding RNAs)	Alix, Tsg101, tetraspanins (CD81, CD63, CD9), flotillin
Microvesicles	50–1000	Plasma membrane blebbing	Proteins, lipids, and nucleic acids (mRNA, miRNA, and other noncoding RNAs)	Integrins, selectins, CD40
Apoptotic bodies	500–2000	Plasma membrane blebbing	Nuclear fractions, cell organelles	Annexin V, phosphatidylserine

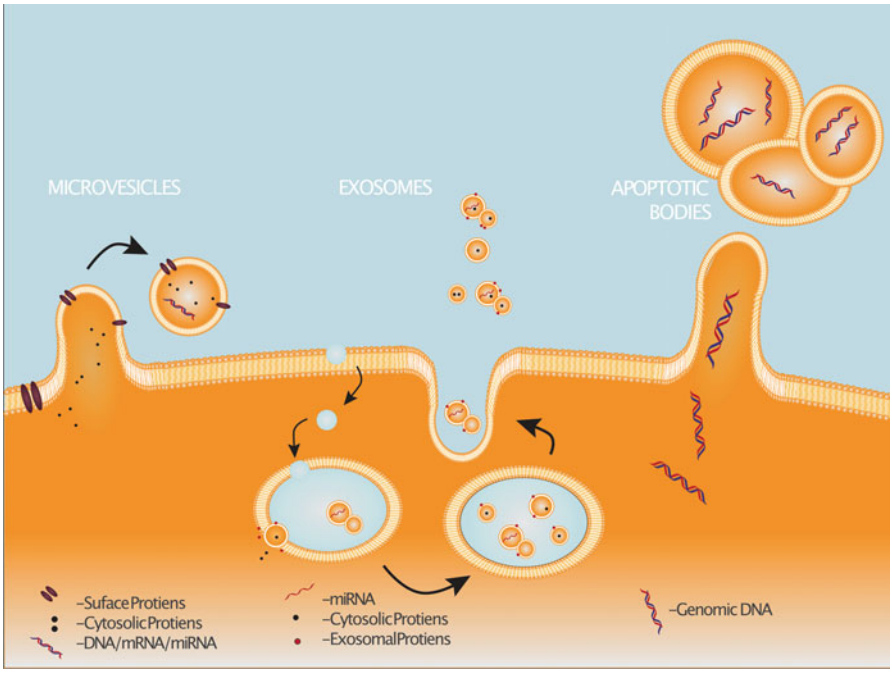

Fig. 2 Extracellular vesicles are heterogeneous. The major populations include exosomes, microvesicles, and apoptotic bodies. Each subtype contains a unique surface architecture and carries distinct cargo. (Used with the permission of Chibuikem Nwizu, chibuikem_nwizu@brown.edu)

Being membrane-bound, they protect their rich cargo from degradation and allow for the shuttling and delivery of selectively packaged proteins, various types of RNA, bioactive lipids, and DNA (Borgovan et al. 2019). As highlighted in Fig. 3, once the vesicles reach a target cell, they impart various long-lasting downstream phenotypic and genotypic effects, which are likely mediated via intricate genetic and epigenetic variables.

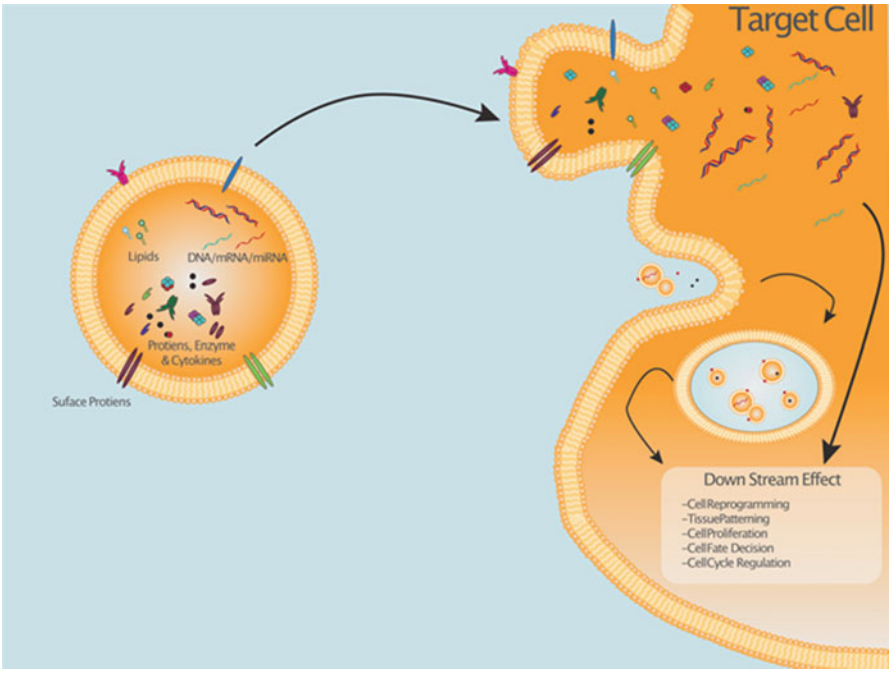

Fig. 3 Extracellular vesicles contain a large array of biologically active cargo. Once the vesicles reach the target cell, the released cargo imparts numerous downstream effects. (Used with the permission of Chibuikem Nwizu, chibuikem_nwizu@brown.edu)

Initial studies focused on EVs functional mechanisms have established the ability of vesicles to directly transfer protein into target cells, while modifying the phenotype of the recipient cells. The initial mediators of EVs' function were primarily hypothesized to be protein and mRNA effectors. The biological effects of vesicles are inhibited after heat inactivation or pretreatment with RNase, suggesting the relevant involvement of protein and mRNA. However, the downstream functional and phenotypic effects observed in EV studies are persistent and long-lasting, spanning well beyond the degradation time expected for biologic proteins, thus suggesting other mechanisms at work. While most plasma miRNAs are bound to proteins, there is a smaller amount associated with extracellular vesicles. Further analysis has indicated that cellular phenotype alteration may be mediated by the transfer of transcriptional activators, possibly miRNA, and downstream modulation of epigenetic signals (Aliotta et al. 2015).

Ratajczak et al. (2006) elegantly highlighted that embryonic stem cell-derived microvesicles successfully reprogrammed hematopoietic progenitors by horizontal transfer of their mRNA and protein payload (Borgovan et al. 2019). The analysis of EVs from embryonic stem cells has identified the expression of stem cell-specific molecules with established roles in stem cell proliferation and self-renewal.

These vesicles also upregulated the expression of various early pluripotent HSCs-specific markers, including Oct-4, Nanog, Scl, HoxB4, and GATA 2.

This mechanism of action was also supported by other research groups, highlighting RNA transfer and phenotypic change in different experimental models (Aliotta et al. 2006, 2007, 2015; Valadi et al. 2007). In these experiments, a genomic change could only occur once the vesicles had entered the target marrow cells allowing for the transfer of both mRNA and transcriptional regulators. As in other studies, the phenotypic effects were long-lived, well beyond the degradation of the transferred mRNA, once again suggesting that long-term expression of downstream proteins derived from the target cells (such as surfactants B and C, which were tested in these studies) represented a stable downstream epigenetic event (Aliotta et al. 2015).

Extracellular Vesicle Pleiotropy and Regenerative Potential

EVs can be successfully isolated from virtually all bodily fluids and cells. EV populations harbor a functional endpoint specific to originator cell type and disease state. There are far-reaching implications in utilizing EVs toward clinical endpoints focused on disease identification, progression, and modulation. The recent focus has been on the capacity of EVs to restore injured tissue and treat disease (Wen et al. 2017). EVs packaging and cargo composition are constantly cycling, and it is in flux with the ever-changing homeostasis of the host's cellular and physiological environment. As a result, EVs selectively package their cargo to address the functional endpoints at hand specifically. Moreover, as we will come to explore, EVs harness a potent regenerative ability in various disease states, including multiple types of solid and hematological malignancies, and can have direct protective and regenerative effects on perhaps the most important of cell regulators – the HSCs.

Extracellular Vesicles Affect Normal Stem Cells

MSCs reside in the bone marrow and adipose tissue. They have multipotent differentiation capacity and, via direct co-transplantation, have been shown to support the HSCs directly by enhancing engraftment and improving bone marrow recovery from radiation in NOD/SCID mice. Wen et al. demonstrated that the functional mediators of these regenerative effects were attributed to the released EVs (Wen et al. 2016). Through elegant studies, his group successfully demonstrated the capacity of human MSCs-derived EVs to reverse radiation-induced damage to the bone marrow and gastrointestinal tissues of mice. EVs demonstrated a capacity to reverse radiation damage to the marrow and gastrointestinal tissues of mice, with the most potent effect on long-term engrafting stem cells (Wen et al. 2016). These results are in accordance with numerous other studies demonstrating how EVs can target and salvage damaged stem cells, promoting their differentiation, and proliferation, thereby reversing radiation-induced damage and apoptosis of the bone marrow cells.

A common functional theme was once again observed in this interaction wherein mRNA was vital. Overexpression of multiple RNA populations involved in cell recovery, growth, radiation resistance, and promotion were observed, allowing for more efficient DNA repair and downregulation of apoptotic factors. Many of these effects were found to be closely integrated into the cell cycle status. In line with the stem cell continuum postulate described earlier in the chapter, Aliota et al. showed the functional effects of vesicles on marrow mRNA expression depended upon the cell cycle status of the target bone marrow cells and the condition of the originator lung cells, in this case either irradiated or not (Aliotta et al. 2012). The results showed that lineage depleted, Sca-1+ murine bone marrow cells showed peak pulmonary epithelial cell-specific mRNA expression in cell cycle phase G0/G1 when the vesicles were derived from irradiated lung tissue, while the peak was in the late G1/early S phase when the vesicles were derived from the nonirradiated lung.

Extracellular Vesicles Affect Cancer Stem Cells

We have explored the evidence illustrating the bimodal interplay among stem cells and EVs. Unique populations of vesicles are released by healthy dividing stem cells (such as the MSCs described above), which in turn are received and employed by other stem cells, which may be stressed or damaged, toward a regenerative purpose (Wen et al. 2016). The vesicle "fingerprint" utilized by stem cells is subject to multiple variables as described in the studies above, including the tightly regulated and continuous phenotypic and genotypic flux the stem cell experiences as it moves through the cell cycle. Much of the reversible changes in short- and long-term engraftment, progenitor numbers, gene expression, and differentiation potential explored in the aforementioned studies are partly a direct result of EVs' effects at various cytokine-induced cell cycle transits (Colvin et al. 2010, 2007; Goldberg et al. 2014). The regenerative potential of stem cells in which there is an injury-related conversion of bone marrow-derived stem cells toward different tissue cells in order to reconstitute damaged populations is, again, largely dependent on EV manipulation of the stem cell continuum during cell cycle. For instance, damaged irradiated lung tissue releases EVs which can transit to and enter the bone marrow stem cells, thereby modulating the stem cell continuum, driving stem cell differentiation, and leading to the bone marrow cell expression of lung-specific mRNA and protein (Aliotta et al. 2015).

The fundamental postulates of the stem cell continuum theory and its applicability to healthy functional stem cells can be accurately extended to the biological mechanics that drive other "types" of stem cells. Cancer stem cells (CSCs) compose a relatively dormant subset of pluripotent cancer cells that have preserved the ability to differentiate, self-renew, and remain particularly resistant to chemo- and radiotherapeutic intervention (Hervieu et al. 2021). These cells have enhanced tumorigenicity and continuously adapt to the myriad of immunogenic and therapeutic threats

they are taxed with, in order to maintain a pro-oncogenic microenvironment and ensure cancer growth (Margolin et al. 2015).

Akin to their HSCs counterparts, CSCs are in a dynamic state of continuum, perpetually changing to respond adequately to its environment – this modulation is thought to be in part regulated by the effects of EV production and reprogramming. Kim et al. followed the expression of various CSCs-specific markers postulating that these aberrant CSCs were constantly reprogrammed during oncogenesis (Kim et al. 2013). Su et al. described these adaptations as a "continuum state," showing that CSCs could transiently cycle through phenotypic (and likely epigenetic) states, with nearly 75% of their surface markers shared by adult or embryonic stem cells. Moreover, the remaining surface architectural composition showed significant variability and cycling, with the majority of these markers being found on differentiated normal tissue cells (Su et al. 2021). Mani et al. highlighted a similar link between EVs and the dedifferentiation process that a mature (or progenitor) cell undergoes when it acquires "tumor-initiating" properties (Mani et al. 2008). Both the research groups evidenced a critical role of EVs in reprogramming stem cell fate and modulating its microenvironment and stroma to harbor cancer growth. When following the expression of variable CSCs-specific markers, Wang et al. postulated that the observed phenotypic changes were linked to, and dependent on, EV manipulation (Wang et al. 2007). The effects were observed as bidirectional, with aberrant (cancer) stem cells promoting a microenvironment of the specific phenotypic state that contributed to the biogenesis, loading, and special packaging of EVs which promote CSCs propagation and further enhance oncogenesis.

An Introduction to Extracellular Vesicles and Hematological Malignancies

EVs play an essential role in intracellular communication through active transcellular crosstalk and post-apoptotic residual messaging (Borgovan et al. 2019). The role of EVs in CSCs' promotion, cancer pathogenesis, and their use in diagnosis, prognosis, and treatment remains an area of immense ongoing research. Herein, we will review the ongoing research surrounding EVs in hematologic malignancies with a special focus on the preclinical data exploring the role of the bone marrow tumor microenvironment and the utility of EVs in diagnostic and prognostic models.

Extracellular Vesicles in Leukemia

The Leukemic Bone Marrow Microenvironment

Leukemia is defined by the development of a clonal proliferation of malignant hematologic cells, with frequent bone marrow infiltration, as well as peripheral blood involvement. Recent research has expanded on this definition and identified

some critical interactions between leukemic cells and the bone marrow microenvironment in cancer development and proliferation. Specifically, it is theorized that leukemic cells induce changes in the bone marrow microenvironment which are more favorable to leukemic cells' growth and proliferation and less conducive for normal hematopoiesis (Duarte et al. 2018). EVs are thought to be one mechanism by which leukemic cells can induce microenvironmental changes (Kumar et al. 2018). Kumar and colleagues demonstrated that treatment of experimental mice with acute myeloid leukemia (AML)-derived exosomes successfully induced similar bone marrow changes in the recipient animals as were caused by typical AML cell engraftment in these mice populations.

The exact mechanism by which leukemic EVs manipulate the bone marrow microenvironment remains less well-explored. In vitro studies with various AML cell lines and the plasma from mice-bearing AML xenografts have shown that the diseased animals released leukemic EVs laden with multiple stem cell-related microRNAs that target the downstream transcription factors involved in stem and progenitor cell growth and differentiation. Through the EV-directed epigenetic changes, AML can inhibit specific stem cell transcription factors with established roles in malignancy and other similar processes, necessary for the proper development of B-cell precursors (Borgovan et al. 2019). Peinado and colleagues demonstrated that melanoma-derived EVs would transfer the receptor tyrosine kinase MET, inducing its expression on bone marrow progenitor cells, which might create a more favorable metastatic environment (Peinado et al. 2012). Huan et al. identified similar bone marrow manipulation by AML cell lines. They demonstrated that AML cells transferred mRNA via EVs to the bone marrow stromal cells, thereby altering the biologic functions of these stromal and hematopoietic cells to create a leukemic microenvironment that would be more conducive for malignant growth (Huan et al. 2013).

This intra-environmental EVs-based communication is not limited to AML. For example, Corrado et al. identified a similar paradigm in the growth and survival of chronic myelogenous leukemia (CML) cells (Corrado et al. 2014). Based on the published data, IL-8 plays a critical role in CML growth and survival, and treatment of the bone marrow-derived stromal cells with CML-derived EVs significantly increases the production of IL-8.

Predictors of Response and Promoters of Resistance in Leukemia

While research is ongoing to identify further the role of EVs in the development and maintenance of bone marrow microenvironment homeostasis and as an emerging novel target for therapeutic intervention, preliminary data have demonstrated a potential role for leukemic cell-derived EVs in prognosis. Hong et al. measured the TGF-β1 level in EVs derived from patients with newly diagnosed AML and followed its level through the course of treatment (Hong et al. 2014). They found that reduction in the EV-derived TGF-β1 level was positively correlated with AML blast reduction during the treatment. While the TGF-β1 level was not predictive of relapse during consolidation, the level in patients with long-term remission (>2 years) was shown to be similar to control patients not diagnosed with AML.

EVs play a critical role in predicting treatment response and are also explicitly involved in promoting drug resistance. Bouvy et al. showed the transfer of chemotherapy resistance between promyelocyte leukemic cell lines via direct cellular transfer of EVs (Bouvy et al. 2017). Chemosensitive cell lines treated with EVs derived from chemoresistant cell lines were shown to endocytose EVs into their intracellular compartments. The recipient cells subsequently showed increased resistance to daunorubicin compared to the untreated chemosensitive cells. A similar study was conducted by Bebawy et al. in which EVs from resistant cell lines, especially those with a high level of P-glycoprotein expression, were cultured with chemosensitive cells. The co-cultured chemosensitive cells subsequently not only expressed P-glycoprotein but also developed its functional mechanisms (Bebawy et al. 2009).

Total plasma EVs isolated from leukemic patients may be exploited as a distinct biomarker. Machine learning-based algorithms have been employed to classify diverse leukemic EVs' populations. Using these mathematical and statistical models to quantify and qualify various EV populations from a heterogeneous patient population at different stages of treatment has shown promising preliminary results toward the EVs use as theranostic biomarkers for both diagnoses and gauging therapeutic response (Borgovan et al. 2019).

Extracellular Vesicles in Lymphoma

The Lymphoma Tumor Microenvironment

The tumor microenvironment in lymphoma allows the growth and proliferation of malignant lymphocytes, a process facilitated by EVs-directed transcellular communication. Hansen et al. identified CD30 ligand expression in Hodgkin's lymphoma-derived EVs, and these EVs were associated with the stimulation of IL-8 release, a pro-inflammatory cytokine implicated in the tumor growth (Hansen et al. 2014). They further demonstrated that CD30+ EVs formed a communication complex between the scattered malignant Reed-Sternberg cells and the surrounding immune meshwork. The group theorized that this might explain the therapeutic benefits of brentuximab vedotin, the antibody-drug conjugate directed against CD30, and that despite the meager propensity of CD30+ cells, they might be just a part of a more integrated EVs-mediated communication network supporting malignant cell growth and survival. On the same note, Dorsam et al. demonstrated how Hodgkin's lymphoma-derived EVs communicated and manipulated the tumor microenvironment (Dörsam et al. 2018). They also documented the uptake of Hodgkin's lymphoma-derived EVs by surrounding fibroblasts and significantly impacted fibroblasts' migration. Uptake of EVs by the fibroblasts also resulted in a phenotypic change producing more cancer-associated fibroblasts. These findings again pointed to an elaborated network of communication and regulation within the tumor microenvironment in which EVs played a unique and central role.

An Introduction to Extracellular Vesicles and Breast Cancer

Breast cancer is one of the most common invasive cancers in women (Desantis et al. 2014). Despite advances in breast cancer therapy, the overall 5-year survival for patients with metastatic breast cancer (MBC) is only 28% (Desantis et al. 2014; Siegel et al. 2021). The clinical course and progression of breast cancer is a dynamic process and involves close interaction with the tumor microenvironment. There is a constant acquisition and regulation of signals, intercellular communication, as well as genetic and epigenetic alterations between cancer cells. Thus, the drivers that boost cancer evolution are essential in understanding and investigating disease progression and novel therapies.

As discussed in the hematologic malignancies section, EVs are biomolecules released by cells to act as a carrier of various biomolecules to facilitate intracellular signaling and homeostasis between the communicating cells. Such cell-to-cell communication has been observed extensively in various solid tumor cell types, including breast cancer.

Role of TEVs in Breast Cancer

Tumor-derived EVs (TEVs) can be retrieved from various bodily fluids to serve as potential biomarkers for theranostic applications (Kalluri 2016; Théry et al. 2002; De Toro et al. 2015; Van Niel et al. 2018). Studies have shown that TEVs play a critical role in metastasis, progression, and tumor initiation (Abak et al. 2018). TEVs produced from cancer cells have been shown to induce proliferation of the neighboring normal cells besides their ability to induce malignant transformation, proliferation, and oncogenic amplification (Jing et al. 2013).

TEVs also help cancer cells evade immune surveillance and promote therapeutic resistance by regulating drug sensitivity via various modalities (Lowry et al. 2015). This extends beyond the standard chemotherapeutic resistance to immunotherapy. EVs have numerous suppressive effects across the population of immune cells that resides in the tumor microenvironment (Borgovan et al. 2019). Yu et al. have shown that breast cancer-derived TEVs could block the differentiation of myeloid precursor cells into dendritic cells. These data have opened an opportunity to use TEVs in cancer immunotherapy (Yu et al. 2007). As discussed elsewhere in this chapter, uptake of TEVs by the fibroblasts promotes their genetic and phenotypic transformation into cancer-associated fibroblasts, a phenotype known to cause immunosuppression.

TEVs are also crucial in regulating metastases, and hence the treatments targeting TEVs may have significant utility in breast cancer as part of novel therapeutic intervention. Studies have shown TEVs derived from aggressive subclones of the triple-negative breast cancer cell line Hs578T were able to transfer their aggressive phenotype to a panel of breast cancer cells. By silencing one of the central regulators of TEVs secretion in breast cancer (GTPase Rab27a), there were a reduction in tumor growth and a significant decrease in metastatic dispersal (Bobrie et al. 2012). Other data have elucidated that TEVs can regulate the selective tropism of cancer

cells to target specific organs and create sites of metastasis that are pro-oncogenic. This is achieved through differing integrin expressions. TEVs also target organ-specific cells and alter integrin expression in that microenvironment to prepare the pre-metastatic niche.

Various experiments have shown that CSCs and their microenvironment are affected by TEVs. Sansone et al. showed that the transfer of exosomal miR-221 to the luminal breast cancer cells, in the setting of hormonal therapy, induced plasticity, a stem cell-like state, and ultimately led to the development of resistant tumor cells (Sansone et al. 2017).

TEVs have an impact not only on tumor promotion but can also inhibit tumor growth. We have explored the role of MSCs in the leukemic niche and have shown that EVs derived from healthy MSCs are negative regulators of the cell cycle and inhibit cancer cell growth. Ono et al. showed that bone marrow-derived MSCs secreted EVs, which acted as negative regulators of breast cancer growth (Ono et al. 2014). These findings are supported by various research groups who have reported that EVs from otherwise healthy (noncancerous) cells can promote breast cancer cell dormancy in metastatic sites (Bruno et al. 2013).

Use of TEVs in Breast Cancer Treatment

TEVs are membrane-encapsulated small vesicular structures and have the ability to maintain their structural integrity and protect their cargo against external processes, such as proteases and other enzymes. We will review how TEVs can become a novel candidate as a "drug-loaded" delivery system for targeted cancer gene therapies (Théry et al. 2002). Various experimental studies have demonstrated that TEVs can be secreted at higher levels in the setting of cytotoxic chemotherapy and enhance the pro-metastatic capacity of cancer cells and allow for chemoresistance through the release and transfer of TEVs from chemoresistant to chemosensitive breast cancer cells (Keklikoglou et al. 2019; Wang et al. 2017).

Conversely, TEVs have also been shown to play a role in overcoming treatment failure due to drug resistance in breast cancer. Some studies have also demonstrated a potential clinical use of TEVs to treat chemoresistant cancer cells. Zhang et al. (2015) highlighted the importance EVs have in the modulation of miRNA expression profiles that led to the reversal of drug resistance via the EVs-directed changes in multidrug resistance-related miRNAs. Researchers have postulated that manipulating drug efflux from the cells may successfully restore drug sensitivity in cancer and CSCs. This was confirmed by Kong et al. in the human breast cancer MDA-MB-231 cell line (Kong et al. 2015).

TEVs have also been investigated for targeted treatment of breast cancer based on their ability to deliver a specific cargo of bioactive molecules into the tumor microenvironment. Thus, they can potentially increase the option of targeted therapy to specific areas using donor-derived products as vectors. In theory, donor cells can produce TEVs with little or no immunogenicity and stable enough to be protected from destruction during delivery.

The potential of TEVs as a delivery vehicle for certain molecules has been assessed in clinical settings. For example, Phase I clinical studies have used dendritic cell-derived TEVs to treat patients with various solid cancers (Escudier et al. 2005; Morse et al. 2005). In addition, Shin-Ichiro et al. have successfully demonstrated the use of breast cancer-derived exosomes to deliver specific miRNAs to EGFR-positive cancer cells (Shin-Ichiro et al. 2013). Many studies intend to use TEVs to encapsulate anticancer drugs/chemotherapeutic agents, such as paclitaxel and doxorubicin, for delivery across the blood-brain barrier and into the CNS (Yang et al. 2015).

TEVs-mediated drug delivery has a broad range of future clinical applications and offers a new treatment strategy in targeting specific pathways such as HER2, PI3K/AKT, and VEGF via direct and indirect modulation of these cellular pathways (Wang and Gires 2019). One example of TEVs application in the setting of targeted therapy is with HER2-expressing breast cancer cells. Studies showed that EVs released by HER2-positive breast cancer cells could bind with trastuzumab and resulted in the suppression of drug effects. These data indicated that TEVs could disrupt anticancer therapy, such as trastuzumab, and promote drug resistance (Marleau et al. 2012). However, by removing exosomes through the creation of a novel therapy called HER2osome (Aethlon Medical, San Diego, USA), which aims to reduce the quantity of circulating HER2 protein and breast cancer exosomes, the therapeutic effects of trastuzumab can be restored.

Use of TEVs for Diagnostic and Predictive Markers in Breast Cancer

Given that TEVs play a vital role in tumor development and metastases, they have been studied for use as diagnostic and prognostic markers. Furthermore, there is promising data that TEVs can be detected before tumor detection or symptomatic appearance of the disease (Saraswat et al. 2015). Thus, the use of TEVs as biomarkers is a promising approach as it may serve as a minimally invasive diagnostic tool due to TEVs availability in various body fluids. Moreover, TEVs can be easily measured during course of treatment as a predictive biomarker for assessing response.

Various research groups have reported specific TEVs associated with distinct disease phenotypes. For example, exosomal miR-373 and miR-939 are linked with triple-negative breast cancer and aggressive breast cancer phenotypes (Corinna et al. 2014; Di et al. 2017). Although the best modality for detecting and analyzing these TEVs is under investigation, high-sensitivity PCR and next-generation sequencing are emerging as promising possible options for their detection.

There has been a significant advancement in breast cancer treatment over the last decade, with TEVs-based diagnostic and therapeutic applications giving encouraging results. Various research groups are analyzing a relationship between TEVs and breast cancer cells regarding their role in cell proliferation, invasion, and metastasis. TEVs are unique in their ability to transport bioactive molecules with protection from degradation both locally and throughout the body.

An Introduction to EVs and Lymphoma

There are over 80 types of lymphomas, including both Hodgkin's lymphoma (HL) and non-Hodgkin's lymphomas (NHL) of B-lymphocyte and T-lymphocyte in origin, as well as indolent and aggressive lymphomas with an expanded spectrum. Even among the established lymphoma subtypes, there exists considerable genetic heterogeneity. Similar to the nature of the disease, therapeutic options and the standard clinical course also have vast heterogeneity. Standard therapy for the majority of lymphomas includes chemotherapy, and in B-cell lymphomas it is typically combined with an immunotherapy (such as the monoclonal anti-CD20 antibody, rituximab). In the treatment for aggressive lymphomas, therapy is of definitive intent, whereas in indolent lymphomas, patients can expect to have periods of therapy and remission. Research on how EVs may better guide diagnosis of lymphoma, assess and predict response to therapy, and ultimately serve as a therapeutic agent is underway.

Extracellular Vesicles in Primary CNS Lymphomas

As in the case of other diseases and in normal physiologic states, lymphoma-derived EVs contain cell-specific cargo of bioactive molecules that includes DNA, RNA, and proteins (Navarro-Tableros et al. 2018; Trajkovic et al. 2008). Lymphoma-derived EVs and parent cells from which they originated express many common surface markers, such as CD19, CD20, and CD30 (Trajkovic et al. 2008; Yao and Wei 2015). As mentioned, compared to healthy tissues, cancer cells show increased release of EVs, making them particularly abundant in the disease state (Yu et al. 2005; Van Eijndhoven et al. 2017). EVs generally contain shared genomic materials, the majority of which is double-stranded DNA, but their cargo also includes numerous proteins, bioactive lipids, and RNA species that are capable of entering into target cells to alter the transcription and expression of genes and proteins related to numerous cellular functions (Navarro-Tableros et al. 2018). All of these aspects make the study of EVs integral to improved diagnosis, prognostic modeling, and possibly treatment of lymphoma.

The Role of EVs in Diagnosis and as a Biomarker in Primary CNS Lymphoma

Liquid biopsies and cell-free DNA analyses continue to gain increasing relevance across several hematologic and solid malignancies. In lymphoma, excisional lymph node biopsy, when feasible, remains standard of care. With improved techniques and optimal protocols, both by interventional radiology and pathology, core biopsies will often suffice. However, with any biopsy, there is a relatively increased risk and discomfort to the patient due to the invasiveness of the procedure when compared to drawing a blood sample for use. In addition, sequential biopsies are at times

necessary to confirm either response or progression but cannot occur routinely throughout treatment as a marker of response.

Cell-free DNA sampling has emerged as a potential tool for assessing treatment response in DLBCL (Kurtz et al. 2015, 2018). Indeed, any liquid biopsy may serve as a biomarker of therapy response, ideally more accurate than imaging, or as a diagnostic tool when the proposed biopsy carries increased risk. An example would be in primary central nervous system lymphoma (PCNSL) and the morbidity associated with a brain biopsy or even lumbar puncture. Lymphoma-derived EVs make an appealing biomarker for liquid biopsy. They are abundantly present in most body fluids, including not just blood but cerebral spinal fluid, urine, peritoneal fluid, amniotic fluid, and even breast milk (Ofori et al. 2021). Of particular interest and a way to obviate a brain biopsy is the presence of neural-derived EVs in saliva (Guedes et al. 2020). Data from traumatic brain injuries demonstrate that the blood-brain barrier does not prevent EVs originating from the CNS from entering the saliva, and potential exists for EVs as a diagnostic tool in CNS malignancies (Guedes et al. 2020; Ramirez et al. 2018; Cheng et al. 2019, 2020).

The use of EVs as the target in a liquid biopsy can also be helpful to discriminate between false-positive and false-negative results besides differentiating unclear diagnoses (Caivano et al. 2015). For example, HL-derived EVs express high levels of CD30 in contrast to CD20 and CD22 in the case of NHL. Tracking of CD20+ EVs also correlates well with the volume of B-cell lymphomas (Domnikova et al. 2013). In general, the actual number of circulating EVs has been correlated with disease progression in HL (Provencio et al. 2017).

An elegant study by the Spanish Lymphoma Oncology Group assessed mRNA for C-MYC, BCL-XL, BCL-6, NF-κβ, PTEN, and AKT in EVs from B-cell lymphomas (Provencio et al. 2017). The authors concluded that the presence of BCL-XL mRNA and its increased level in BCL-6 mRNA in EVs might serve as suitable measurement targets for response and surveillance.

Extracellular Vesicles' Function on Lymphomagenesis and Lymphoma Treatment Response

EVs can enhance, but primarily impair, the immune response to malignancy through multiple methods, including effects on both innate and adaptive immunity (Navarro-Tableros et al. 2018; Théry et al. 2009). This has wide-ranging implications in disease progression and the efficacy of various therapies to target disease. EVs' inhibition of regulatory T-cells (T-regs) plays an essential role in the immune escape of lymphomas (Navarro-Tableros et al. 2018). In doing so, T-regs decrease the efficacy of lymphoma-infiltrating T-cells and impair the immune response in HL (Marshall et al. 2004). In addition, EVs from EBV-positive lymphomas have been shown to incorporate into macrophages and induce an immune regulatory phenotype (Navarro-Tableros et al. 2018). PD-L1-expressing EVs suppress T-cell response within the lymph nodes (Poggio et al. 2019). EVs can also alter the function of

natural killer (NK) cells in lymphoma (Navarro-Tableros et al. 2018). NK cells express the natural killer group 2, member D (NKG2D) receptor that plays a role in recognizing stressed self-molecules, similar to PD-1 in the T-cells (Nausch and Cerwenka 2008). EVs increase the NKG2D ligand, thereby activating a more robust immune response (Navarro-Tableros et al. 2018). In addition to supporting immune surveillance, EVs promote angiogenesis in lymphoma (Navarro-Tableros et al. 2018). Lymphoma-associated EVs deliver vascular endothelial growth factor (VEGF) and other angiogenic proteins within the lymphoma microenvironment (Yang et al. 2015; Chen et al. 2018). Regarding the treatment response, EVs also express CD20 and may "trap" antibodies relevant in therapy (i.e., rituximab) and thereby dampen the therapeutic effects (Navarro-Tableros et al. 2018; Aung et al. 2011).

EVs' Effects on Lymphoma Patient Outcomes and Prognosis

EVs have been studied as prognostic markers in lymphoma. Expression of EVs affecting specific markers, including HSP-70, c-Myc, Bcl-2, Mcl-1, xIAP, and Bcl-xL, has been associated with impaired response to therapy in aggressive lymphomas (Chen et al. 2018). In a study by the Spanish Lymphoma Oncology Group, expression profiling of multiple mRNAs in EVs had prognostic implications. EVs with AKT mRNA demonstrated an inadequate rituximab response (Provencio et al. 2017). High death rates were also observed in the presence of BCL-6 mRNA. Additionally, C-MYC mRNA predicted a short duration of response in follicular lymphoma. Like other malignancies, lymphoma patients are at increased risk of thrombosis, and EVs have been postulated to drive this hypercoagulable state (Navarro-Tableros et al. 2018; Litwińska et al. 2019). Systemically, EVs shed by the cancer cells are able to promote thrombus formation via the expression of tissue factor, which is a potent trigger of the coagulation cascade and thrombotic events. Moreover, various cancer and oncology treatments lead to the release and activation of platelet-derived EVs, further stimulating vascular remodeling and clot formation (Borgovan et al. 2019).

Role or Target in Therapy for Lymphoma

As EVs clearly promote lymphomagenesis and resistance to therapy, therapeutic interventions directed toward lymphoma-related EVs create a novel avenue of research. As additional B-cell-specific antibodies are being studied or as in the case of anti-CD19 tafasitamab that has been already approved, their combined use may neutralize lymphoma-derived EVs and allow better therapeutic efficacy (Navarro-Tableros et al. 2018; Salles et al. 2020). Another approach is to block the release of EVs from lymphoma cells. As the generation and release of EVs are complex processes, targeting their formation and release by lymphoma cells presents

a challenge. Several imidazoles and other agents interfere in pathways associated with EVs biogenesis and release (Datta et al. 2018). Other promising agents can impair Ca2+-dependent release of EVs (Savina et al. 2003).

Additionally, EVs also provide a way to immunogenically prime and "educate" dendritic cells, which in turn could create T-cell expansion against various lymphoma epitopes and ultimately an anti-lymphoma response (Litwińska et al. 2019). Furthermore, dendritic cell-derived EVs-based methods have shown promise in mouse models when combined with multiple standard lymphoma therapies, such as cyclophosphamide (Taieb et al. 2006). Finally, dialysis-type approaches have been proposed to physically remove EVs, although no models have shown efficacy in malignancy so far (Navarro-Tableros et al. 2018).

Lymphoma-Specific EV Roles

Diffuse Large B-Cell Lymphoma (DLBCL)

The most common NHL, DLBCL, has emerged as a truly heterogeneous disease. Gene expression profiling first defined major subgroups, germinal center B-cell (GCB), activated B-cell (ABC), and not-classifiable more than two decades ago (Alizadeh et al. 2000). Since that time, immunohistochemistry-based algorithms have attempted to capture these groups with more straightforward techniques to varied success, with the Hans algorithm being the most accepted algorithm type (Hans et al. 2004). Several groups have recently used molecular profiling to define several more distinct subgroups, each with their prognosis, and possibly distinct treatment vulnerabilities (Chapuy et al. 2018; Reddy et al. 2017; Schmitz et al. 2018). EVs may serve as an adjunct to this taxonomy or may even offer a new, improved method for delineating DLBCL-specific gene expression without the need for biopsy (or repeat biopsy) and provide insight into therapeutic selection. Feng and colleagues using cell samples derived from patients undergoing treatment for diffuse large B-cell lymphoma found a specific high-level miRNA expression profile in EVs of patients with the chemoresistant disease compared to those with the chemoresponsive disease. They showed a clear difference in progression-free survival when patients were stratified based on low or high expression of this specific miRNA marker in EVs (Feng et al. 2019). Zare et al. showed a similar analysis of miRNA, demonstrating a significant difference in EVs levels in patients with refractory or relapsed disease compared to those who responded to standard chemotherapy (Zare et al. 2019).

Virally Mediated Lymphomas

In addition to EBV, Kaposi sarcoma-associated herpesvirus (KSHV), human immunodeficiency virus (HIV), hepatitis C virus (HCV), human T-lymphotropic virus-1 (HTLV-1), and others have all been associated with lymphomagenesis through either directly induced transformation, chronic inflammation, or immunodeficiency and associated reduced surveillance (Xu and Wang 2017). In HTLV-1-positive cells, EVs contain the Tax protein, which is considered a main oncogenic protein that

contributes to the development of adult T-cell leukemia/lymphoma (Pinto et al. 2019). Much like mammalian cells and bacteria, these viruses can also secrete EVs containing selectively packaged viral mRNA and oncogenes within the lymphoma microenvironment (Xu and Wang 2017). It appears that EBV+ Burkitt's cells secrete pro-angiogenic EVs, unlike EBV- Burkitt's cells which do not secrete pro-angiogenic EVs (Xu and Wang 2017; Yoon et al. 2016). Overall, EVs play an essential and central role in virally mediated lymphomas.

Hodgkin's Lymphoma (HL)

HL is a lymphoma characterized by scattered malignant Reed-Sternberg cells in an inflammatory milieu. EVs constitute a key component of this unique microenvironment. It is generally observed that direct contact dependent cell-to-cell communication supports tumor growth within Hodgkin's lymph nodes. This current dogma is expanding as we begin to understand how EVs can provide a means of efficient noncontact dependent cell communication (Hansen et al. 2014). Surveillance in HL can be difficult, with positron emission tomography (PET) scans showing notably false positives during and after treatment. EVs-associated microRNAs in HL track well with treatment, response to treatment, and long-term follow-up. They can therefore serve as novel tumor biomarkers for response and surveillance. Pegtel et al. evaluated circulating EV-bound miRNAs in the plasma of HL patients, elucidating a significant decrease in various lymphoma-relevant miRNAs, which correlated well with the clinical metabolic response observed via PET. Of note, patients with progressive or relapsed disease had increasing levels of these circulating EV-bound miRNAs (Van Eijndhoven et al. 2017).

A better understanding of the role of EVs in lymphoma offers hopes of ultimately improving patient care and outcomes through several methods. First, when considered as a form of liquid biopsy, lymphoma EVs may obviate the need for difficult or repeat biopsies, help clarify an unclear diagnosis, track response to treatment, and survey for relapse/recurrence. They may both prognosticate and ultimately guide treatment decisions. Either targeting or using EVs may improve the efficacy of therapy. Finally, with a better understanding of EVs, fundamentals of lymphomagenesis may be better elucidated.

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Toll-Like Receptor 3

43

A Multifunctional Regulator of Mesenchymal Stem Cells

Mohamed Mekhemar, Johannes Tölle, Christof Dörfer, and Karim M. Fawzy El-Sayed

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Abstract

Mesenchymal stem/progenitor cells (MSCs) are prospective cellular candidates for numerous regenerative and immunotherapeutic purposes. Their immunomodulatory potential and multilineage abilities allow them to be deployed for the treatment of various conditions. Nevertheless, depending on the local microenvironment, different biological tasks of MSCs can be adjusted. Toll-like receptors (TLRs) represent

Mohamed Mekhemar and Johannes Tölle contributed equally with all other contributors.

M. Mekhemar · J. Tölle · C. Dörfer
Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian-Albrecht's University, Kiel, Germany

K. M. Fawzy El-Sayed (✉)
Stem Cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian Albrechts University, Kiel, Germany

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important bridges regulating the cross talk between MSCs and their microenvironment affecting diverse biological features of the cells. Toll-like receptor 3 (TLR3), a member of TLR family, recognizes double-stranded RNA (dsRNA) produced by DNA viruses and positive-strand RNA viruses. Its expression has been displayed by MSCs of various sources. Upon ligand identification inside the endosomes, TLR3 oligomerizes and recruits the Toll-interleukin-1 receptor domain-containing adaptor molecule-1 (TICAM-1), which triggers the production of NF-κB, β-catenin, IRF3, and AP-1, leading to the secretion of interferons and other immunomodulatory cytokines and results in pathogen clearance, as well as the recruitment of adaptive immune responses. It may also be involved in the cell-fate determination and cell cycle regulation in different developmental stages of the MSCs. Moreover, it has been shown to improve the therapeutic potential of MSCs by the promotion of multifunctional trophic factors. Priming of MSCs' TLR3 within potential treatment procedures may serve as an additional step enhancing the required biological functions of the cells in different stages of the disease.

Keywords

Stem cells · Toll-like receptor 3 · Stemness · Inflammation · Immunomodulation · Differentiation

List of Abbreviations

AD	Adipose tissue
AKT	Protein kinase B
AP-1	Activator protein 1
AT-MSCs	Adipose tissue mesenchymal stem cells
BM-MSCs	Bone marrow mesenchymal stem cells
C/EPB	Ccaat/Enhancer-binding protein
CFUS	Colony forming units
COX-2	Cyclooxygenase-2
DF-MSCs	Dental follicle mesenchymal stem cells
DP-MSCs	Dental pulp mesenchymal stem cells
DSRNA	Double-stranded ribonucleic acid
G-MSCs	Gingival mesenchymal stem cells
GSK3B	Glycogen synthase kinase 3 B
HGF	Hepatocyte growth factor
HO	Hemoxygenase
IDO	Indoleamine-2,3-dioxygenase
IKK	Iκb kinase
INF1	Interferone type 1
IRAK1	Interleukin-1 receptor-associated kinase 1
IRF3/7	Interferon regulatory factor 3/7
IKB	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
JNK	C-Jun N-terminal kinase
LPS	Lipopolysaccharides

MAPK	Mitogen-activated protein kinase
MIF	Macrophage migration inhibitory factor
MIRNA	Micro-RNA
MKK	Mitogen-activated protein kinase kinase
MSC	Mesenchymal stem cell
MTOR	Mechanistic target of rapamycin
MYD88	Myeloid differentiation primary response 88
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NK-CELLS	Natural killer cells
NM-MSCs	Nasal mucosa MSCs
NO	Nitric oxide
P38	P38 mitogen-activated protein kinases
PAMPS	Pathogen-associated molecular patterns
PD-L1	Programmed death ligand-1
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PMN	Neutrophils
POLY(I:C) HMW	High molecular weight polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PT	Placental tissue
RIP1	Receptor-interacting serine/Threonine-protein kinase 1
SCAP	Stem cells from the apical papilla
S-SCR	Cellular sarcoma molecule
SSRNA	Single-stranded RNA
TAB1/2	Tgf-beta-activated kinase ½
TAK1	Mitogen-activated protein kinase kinase kinase 7
TBK1	Tank-binding kinase 1
TH1	Cd4+ T helper 1
TIR	Toll-interleukin receptor domain
TLR	Toll-like receptor
T-MSCS	Mesenchymal stromal cells from human tonsils
TRAF	Tnf-receptor-associated factor
TRIF	Tir-domain-containing adapter-inducing interferon-B
UCB	Umbilical cord blood
UCB-MSCS	Umbilical cord blood mesenchymal stem cells
WJ	Wharton jelly

Introduction

Mesenchymal stem cells (MSCs) are promising contenders for many practices on the grounds of regeneration and immunotherapy (Han et al. 2019; Almeida-Porada et al. 2020). Their multilineage differentiation ability and immune regulatory aspects permit their potential uses to manage various pathologies and medical conditions (Han et al. 2019; Almeida-Porada et al. 2020). Nevertheless, the local

microenvironment and inflammatory condition can modulate diverse immune-regenerative structures and characteristics of the cells (Han et al. 2019; Almeida-Porada et al. 2020; Li et al. 2019). Certainly, during their clinical application, MSCs may show interactive responses to their milieu through their expressed Toll-like receptors (TLRs). Hence, a comprehension of these TLR-mediated responses of MSCs and their effect on the cells' immunobiology, potential MSC-based therapies can be better controlled and optimized (Abdi et al. 2018; Najar et al. 2017).

TLRs link the acquired and innate divisions of the immune response, detecting pathogen-associated molecular patterns (PAMPs), in addition to the damage-associated molecular patterns (DAMPs). In recent years, ten functional extracellular and intracellular TLRs have been characterized for their functional contribution and regulatory involvement in different cellular processes (Henrick et al. 2019). As identifiers of diverse pathogens, they play a significant role in the progress and healing of inflammatory and infectious conditions (Farrugia and Baron 2017; El-Zayat et al. 2019). TLR3 as a member of the TLR family has the ability to identify DNA viruses' double-stranded RNA (dsRNA) and positive-strand RNA viruses, initiating the acquired immune response and pathogen clearance (Chen et al. 2018; Mekhemar et al. 2018a). MSCs of miscellaneous origins have been shown to express TLR3 in definite patterns (Mekhemar et al. 2018a). Upon activation, TLR3 can control MSCs' various functions including proliferation, immunomodulatory responses, and cell-fate determination (Mekhemar et al. 2020). Recent inquiries have shown that activation of TLR3 by Poly (I: C), a highly specific TLR3 ligand, augmented the immunosuppressive response of gingival mesenchymal stem cells (G-MSCs) (Mekhemar et al. 2018a) and human umbilical cord-derived mesenchymal stem cells (UCB-MSCs) (Zhao et al. 2014). These effects were mediated via downstream microRNA-143 inhibition. However, TLR3 activation also enhanced the production of different trophic factors of MSCs (Mastri et al. 2012), promoting stemness and differentiation-associated factors and modulating the cell-fate determination in several types of stem cells (Mekhemar et al. 2020). These effects are mediated by β-catenin and NF-κB co-activation as well as the regulation of Ca^{2+} signaling (Park et al. 2016; Jia et al. 2015; Yeh et al. 2016; Qi et al. 2014; Katoh and Katoh 2007) in a PI3K-AKT pathway controlled response (Zhu et al. 2019; Joung et al. 2011; Baker et al. 2015).

Priming of MSCs' TLR3 as the primary key of regulation within therapeutic procedures may enhance the required biological functions of the cells in diverse phases of the disease and optimize the understanding of MSC immunobiology.

Mesenchymal Stem Cells

Human mesenchymal stem cells were first described as the plastic-adherent, non-hematopoietic cells, isolated from the bone marrow and able to self-renew and undergo multilineage differentiation in vitro (Friedenstein et al. 1970; Bianco and Robey 2001; Pittenger et al. 1999; Chamberlain et al. 2007). In their undifferentiated

state, these cells can arise from diverse sources within the adult human body (Berebichez-Fridman and Montero-Olvera 2018). While bone marrow is recognized as the primary niche of adult mesenchymal stem cells (Mekhemar et al. 2018b), numerous researchers have tried to find other stem cell sources to achieve large amounts of MSCs with fewer morbidity and risks for the donors. These include placental tissue (PT), umbilical cord blood (UCB), adipose tissue (AD), and Wharton jelly (WJ), which have been defined as dependable niches of MSCs (Wu et al. 2018; Shree et al. 2019; Marino et al. 2019). MSCs can be similarly isolated from oral tissues as alveolar bone proper (Fawzy El-Sayed et al. 2017), gingiva (Mekhemar et al. 2020), periodontal ligament (Banavar et al. 2020), the dental follicle (Hong et al. 2020), and dental pulp (Noda et al. 2019). Despite the phenotypic similarity of MSCs isolated from different tissues, actions and biologic functions have shown differences, accenting the uniqueness of MSCs isolated from each niche (Mekhemar et al. 2020).

MSCs self-renewal describes the process that enables MSCs to increase their number during development. This ability is vital for MSCs to promote their growth inside the tissues and presents a significant contribution in stem-cell-based treatments either alone or in combination with other cell types (He et al. 2009; Haider 2018; Hosseini et al. 2018). This property exhibited its reliance on the cells' span of life, mostly limited to 44 weeks or 55 population doublings in vitro (Mekhemar et al. 2018b). Multilineage ability or multipotency of MSCs labels the potential of the cells to differentiate into other mesodermal cell lineages, including chondrocytes, osteocytes, and adipocytes.

However, they can congruently differentiate to form cells of different embryonic lineages (Uccelli et al. 2008). Recently, MSC identification and "stemness" verification have been a challenging topic in many investigations (Pittenger et al. 2019). In 2006 MSCs' plastic adherence upheld under basic culture conditions was demarcated (Dominici et al. 2006). Furthermore, the multilineage differentiation potential of the cells after stimulation by specialized media was explained by multiple studies (Mekhemar et al. 2018b). Another extensively described method for MSCs identification is the investigation of specific surface marker expression by the cells. Markers as CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120, CD124, CD 146, CD166, CD 271, Stro-1, and SSEA-4 display positive expressions, whereas CD86, CD11, CD80, CD14, CD18, CD31, CD34, CD40, CD45, and CD56 are faintly expressed or completely missing (Lv et al. 2014). Specific cellular colonies shaped by MSCs' culture after isolation were described in further investigations as colony forming units (CFUs), and reported likewise as a method of MSCs' recognition (Mekhemar et al. 2020).

Toll-Like Receptor 3 and Its Activation Pathways

Pattern recognition receptors (PRR) carrying cells are called "sensor cells." The TLRs' expression allows them to recognize various pathogen-associated antigens (Gong et al. 2019). TLRs are single-pass α-helical transmembrane proteins

(Choe et al. 2005). Dimerization strengthens antigen binding and induces signal transduction (Wang et al. 2010). Dimers can either consist of two identical monomers (homodimers, i.e., TLR3, TLR7, TLR8, and TLR9) or two discrete monomers (heterodimers, i.e., TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) (Janssens and Beyaert 2003).

TLRs can be categorized based on two different features, including their localization in the cells (Kawai and Akira 2007b) and connection with intracellular signaling (Takeda and Akira 2004). Concerning localization, TLRs are mainly divided into intracellular or extracellular types. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR 10 are preferentially expressed on the cells surface membrane whereas TLR3, TLR7, TLR8, and TLR9 are mainly carried by intracellular endosomes (Fawzy-El-Sayed et al. 2016). Interestingly, some TLRs, such asTLR3 and TLR4, have the ability to switch their location (Bugge et al. 2017). It is believed that surface membrane expression is accompanied by fastened signal induction. Extracellular TLRs are sensitive to bacterial components like lipopolysaccharides (TLR4), peptidoglycans (TLR1, TLR2, and TLR6) and flagellin (TLR5). TLR10 ligand has not yet been identified, but its heterodimerization with TLR1 and TLR2 implies bacterial substrate, as well (Nagashima et al. 2015). Moreover, endosomal TLRs are nucleic acid sensing in nature and make up a defense against viral attacks. They are sensitive to dsRNA (TLR3), ssRNA (TLR7 and TLR8), and viral DNA (TLR9) (Miyake et al. 2018).

On the other hand, TLRs can be classified based on their adaptor proteins interfacing receptor activation and intracellular signal transduction. Generally, there are two different adaptor proteins: myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). TLR1, TLR2, TLR6, TLR7, TLR8, and TLR10 are only capable of activating MyD88. On the contrary, TLR10 differs from its counterparts as TLR10 does not maintain the adaptor binding determinant. TLR4, TLR5, and TLR9 can use TRIF and MyD88-mediated signaling (Takeda and Akira 2004). TLR3 is the only receptor addressing TRIF standing alone among the other TLRs (Lundberg et al. 2013).

TLR 3 is the first discovered antiviral TLR, which senses viral double-stranded RNA (dsRNA). Alexopoulou et al. (2001) described the induction of transcription factor NF-κB in response to TLR 3 stimulation. They also observed increased production of interferon type 1 (INF1) and various pro-inflammatory cytokines (Alexopoulou et al. 2001). This reaction is now considered one of the crucial and integral part of the antiviral defense and innate immune response activation (Kawai and Akira 2007a).

Consequently, TLR3 is expressed by sensor cells, such as dendritic cells, monocytes, epithelial cells, fibroblasts, and neurons, thus ensuring fast detection of viral invasion (Kawai and Akira 2007a). Apart from that, El-Sayed et al. discovered TLR3 expression in mesenchymal stem cells like G-MSC (Fawzy-El-Sayed et al. 2016), dental pulp stem cells (DP-MSC) (Fawzy El-Sayed et al. 2016), and apical papilla stem cells (SCAP) (Fehrmann et al. 2020). This link between pathogen recognition and tissue regeneration has been proven by Mekhemar et al. (2020). They have

reported a distinct connection between stem cell characteristics and their functionality, and TLR3 ligation.

Primarily, TLR3 has an intracellular location in the endosomes, but some cells, such as dendritic cells and monocytes, have cell membrane surface expression of TLR3 as well. Such altered TLR 3 location renders the cells far higher sensitive for extracellular pathogen recognition and hence provides a potential target for pharmacological immune activation (Murakami et al. 2014).

TLR3 is a single-pass α-helical transmembrane protein segmented into three parts: an extracellular/intraendosomal ectodomain, a transmembrane domain, and an intracellular adaperdomain, and mediates intracellular signal transduction via Toll-interleukin receptor domain (TIR) (Akira et al. 2006). The ectodomain is shaped like a horseshoe containing 23 leucine-rich sequences. Unlike the outer side, the inner side is glycosylated, thus allowing ligand binding on the outer side. After dsRNA binding, one TLR3 monomer forms a fragile complex while a second TLR3 monomer strengthens this connection, thus resulting in the formation of a dsRNA-TLR3 dimer complex (Choe et al. 2005; Wang et al. 2010).

The exact mechanism of intracellular signal transduction remains less well understood. However, changes in TLR3 ectodomain structure during antigen binding or convergence of intracellular domains in dimerization have been extensively studied and discussed in the published literature (Liu et al. 2008; Gosu et al. 2019). TLR3 is the only TLR in which signaling occurs independent of My88 involvement, and it only involves TRIF (TIR-domain-containing adapter-inducing interferon-β).

In trials under TRIF inhibited conditions, Yamashita et al. (2012b) recognized the effects of TLR3 stimulation via dsRNA. This discovery was ascribed to the cellular sarcoma molecule (s-Scr). Apparently, short-time TLR3 activation resulted in c-Scr phosphorylation and induction. On the contrary, longtime receptor stimulation caused c-Scr sequestration and inactivation. Although the exact pathway has not been fully revealed in MSCs, the effect of TLR3 stimulation appears multidirectional (Yamashita et al. 2012b; Mekhemar et al. 2020). Generally, the biphasic effect of TLR3 ligation has been proven in studies on G-MSCs (Mekhemar et al. 2020).

TRIF acts as a checkpoint in TLR3 activation and regulation. TRIF is regulated via K27-linked polyubiquitination via RING E3 ubiquitin ligase complex Cullin-3-Rbx1-KCTD10 complex. Deficiencies result in an obstruction in TRIF-TLR binding and TLR downstream signaling (Wu et al. 2019). TRIF works as a point of origin for four different pathways, finally affecting the transcription factor activation and gene transcription: β-catenin, IRF3/IRF7, NFκB, and AP-1 (Jiang et al. 2004). The distinct transcription factor activation profile depends on various cumulative factors influenced by numerous individually interacting pathways.

As the most critical pathway in MSCs' pluripotency and tumor development, β-catenin activation is influenced by two significant cascades: PI3K/Akt- and Wnt-Pathway. PI3K is induced via TRIF activation that converts the catalytic domain PIP_2 into the activated PIP_3 form. Binding with the PKB/Akt complex enables Akt phosphorylation and activation (Hemmings and Restuccia 2012).

Akt as a kinase is now able to convert cytosolic β-catenin to its active form (Gantner et al. 2012). Akt activation undergoes inhibition via GSK3β but decreases Akt activity in a positive feedback loop. This suppresses GSK3-mediated apoptosis and induces β-catenin-mediated proliferation (Endo et al. 2006). Additionally, Wnt pathway regulates total β-catenin accumulation as well as its nuclear translocation. During Wnt inactivation, β-catenin undergoes inactivation and proteasomal degradation (Fang et al. 2007; Matsumoto et al. 2018; Gantner et al. 2012; Gerlach et al. 2014).

IRF3 and IRF7 are activated via TRIF-dependent TRAF3 activation. This requires a complex of TRIF and TBK1 which impairs K63-linked polyubiquitination of TRAF3 and TRIF (Cai et al. 2017). TRIF/TRA3 associates with IRAK1 and IRF3/7 activating kinases TBK1 and IKKε under TBK, and IRAK1 phosphorylation. This complex then phosphorylates IRF3 and IRF7 monomers. Continuously, both are capable of homo- and heterodimerization ($IRF3_2$, $IRF7_2$, and IRF3/7). Dimers are translocated through nuclear pores primarily inducing INF-1 activation. IRF activation requires PI3K/Akt/mTOR pathway activation. On the one hand, these studies revealed the significance of Akt-mediated phosphorylation of IRF, they also highlight that PI3K is critical in IRF_2 nuclear translocation (Ning et al. 2011) through nuclear pores (Ullah et al. 2016).

NF-κB and AP-1 are induced via TRAF6, wherein NFkB is influenced by TRAF6-TAB1/2-TAK1-RIP1 (Ko et al. 2015). The activity of this complex is guided via ubiquitin-mediated RIP1 regulation (Annibaldi et al. 2018). The IKK complex is now phosphorylated downstream, initiating the breakdown of the NFκB-IκB complex (Kinsella et al. 2018; Abujamra et al. 2006). The freed NFκB is then capable of trafficking into the nucleus, traversing across the nuclear membrane through the nuclear pores as part of the signal transduction. IκB can inhibit NFκB via two primary mechanisms: IκBα follows NFκB into the nucleus and resynthesizes NFκB-IκBα complex, thus resulting in its inactivation. Alternatively, negative feedback proceeds from the fast gene transcription. During RNA accumulation, another transcript IκBδ accumulates, which forms an NFκB-IκBδ complex, thus resulting in an inhibition of the ongoing gene transcription (Mitchell et al. 2016). On the other hand, TRAF6 can induce AP-1 via MAPK pathways and downstream phosphorylation cascades (Roy et al. 2016).

Unlike NFκB activation, AP-1 is not activated via RIP1 induction but depends on GSK3β activity (Ko et al. 2015). It has been reported that GSK3β, TAK 1, and TRAF6 form a ternary complex supporting TRAF6-TAK1-TAB1/2 complex formation. On the contrary, GSK3β did not assemble with RIP1, indicating no influence on NFkB expression (Ko et al. 2015). The TRAF-TAK-TAB-complex phosphorylates MKK3, MKK6, MKK4, and MKK7 using TAK1 (Yamashita et al. 2008). MKK3, MKK4, and MKK6 together initiate p38 MAPK activation, and MKK4/MKK7 induces JNK phosphorylation. JNK and p38 MAPK activation finally result in AP-1 transcription factor activation and gene transcription initiation (Dainichi et al. 2019). Additionally, p38 MAPK modulates C/EPB transcription activity and post-transcriptional protein modification (Dainichi et al. 2019). Figure 1 shows the TLR3 signaling cascades in MSCs after ligation by dsRNA/Poly (I: C) are (Fig. 1).

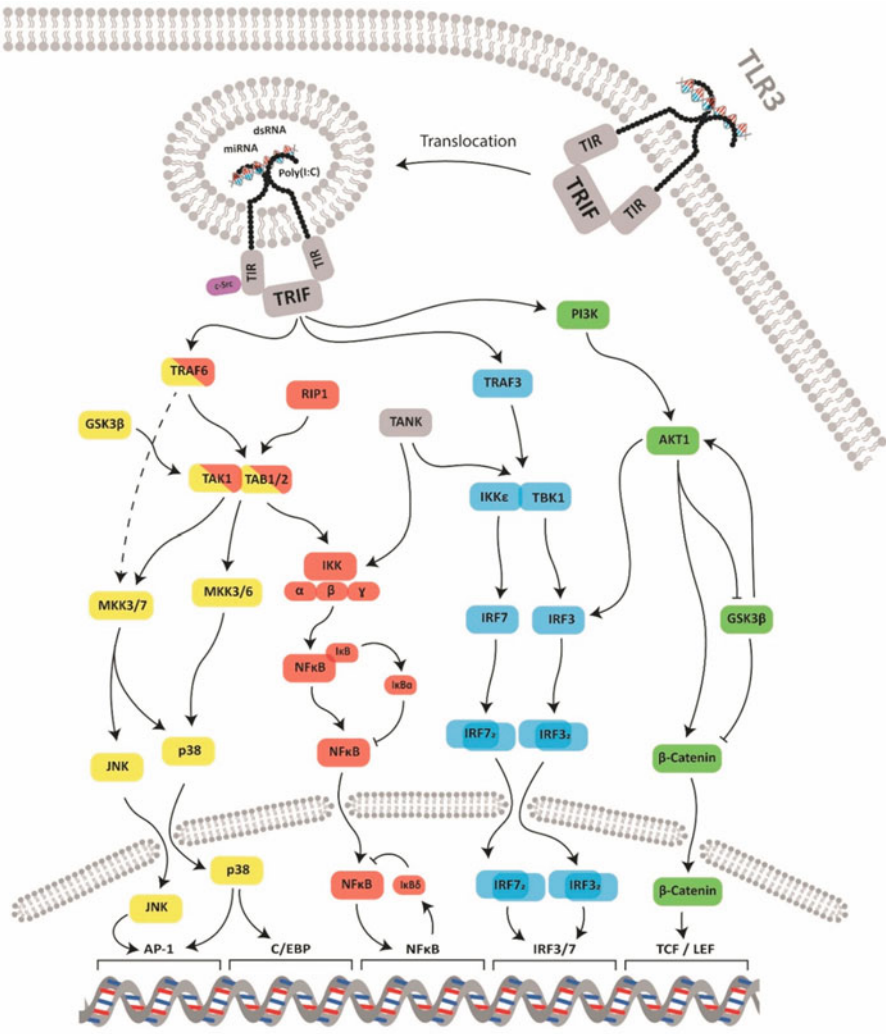

Fig. 1 Graph of TLR3 signaling cascades in MSCs after stimulation by dsRNA/Poly (I: C). TLR3 signals are transmitted to the PI3K-AKT pathway to regulate MSC functions through β-catenin, and to the TRAF pathways to regulate MSC functions via IRF, NF-κB, and AP-1 co-stimulation. TLR3 signaling pathway through the TRIF independent c-Src cascade contributes to the regulation of proliferation in MSCs. (Abe et al. 2020; Arnold et al. 2016; Bai et al. 2014; Baker et al. 2015; Chang et al. 2013; Choe et al. 2005; Dumitru et al. 2014; Fan et al. 2019; Fang et al. 2007; Ferreira et al. 2011; Gambara et al. 2015; Gan et al. 2018; Grandvaux et al. 2002; Harashima et al. 2012; He et al. 2018; Jia et al. 2015; Joung et al. 2011; Katoh and Katoh 2007; Kim et al. 2014; Kim et al. 2006; Krishnamurthy and Kurzrock 2018; Lathia et al. 2008; Leite et al. 2017; Liotta et al. 2008a; Liu et al. 2019; Lombardo et al. 2009a; Lu et al. 2015; Ma and Hottiger 2016; Matsumoto et al. 2018; Najar et al. 2017; Park et al. 2016; Qi et al. 2014; Sallustio et al. 2019; Sarkar et al. 2004; Shang et al. 2017; Wang et al. 2010; Yamashita et al. 2012a; Yamashita et al. 2012b; Yeh et al. 2016; Yoshizawa et al. 2008; Zhang et al. 2013; Zhu et al. 2019). *TIR* Toll-interleukin-1 receptor homology domain, *c-Src* Proto-oncogene tyrosine-protein kinase Src, *TRIF* TIR-domain-containing

TLR3 Priming and MSC Immunomodulation

Recently, the therapeutic abilities of MSCs by modulating immune responses have been reported in different studies (Weiss and Dahlke 2019; Saldaña et al. 2019). The unique interplay between activated MSCs and various immune aspects of the human body contributes significantly to restore and protect damaged tissues in inflammatory environments (Saldaña et al. 2019). TLR-specific ligands have exhibited associations with several inflammatory settings. Stimulation of TLRs is suggested to modify MSCs-regulated immunomodulatory effects. Earlier investigations proposed that the MSCs source and the triggered TLRs play essential role defining these immune biological processes (Mekhemar 2018).

Immune cell migration and binding to MSCs' environment is considered the foremost step for initiating immunomodulation responses after TLR ligation (Najar et al. 2017; Mekhemar 2018). By dynamically identifying their microenvironmental factors via TLR signaling, MSCs can consequently regulate the immune cells' immunobiology from both branches of the immune system (Najar et al. 2017; Mekhemar 2018). This effect initiates the recruitment of MSCs to the injury sites. TLR3 ligation on bone marrow mesenchymal stem cells (BM-MSC) triggered signaling pathways, consequently inducing the cytokine and chemokine secretion that is primarily involved in cell migration (Tomchuck et al. 2008). Indeed, TLR3 ligand exposure promotes the BM-MSC stress migration response and consequently transforms BM-MSCs into strong chemotactic cells, thus enhancing the recruitment of immune-inflammatory cells and endorsing the secretion of cytokines including CCL5 (RANTES), IL-6, IP10, IL-1β, IL-8, and monocyte chemotactic protein (MCP)-1 through the NF-kB pathway (Romieu-Mourez et al. 2009). Previous investigations have also demonstrated that upon TLR3 ligation in BM-MSCs, a Janus kinase (JAK) 2/signal transducer and trigger of transcription (STAT) 1 pathway is started, and cytokine signaling (SOCS) proteins suppressor expression is augmented (Tomchuck et al. 2012). This suggests that TLR3 signaling can noticeably affect the MSCs' response to danger signals and homing-in to injured sites. Moreover, Poly (I: C) activation of TLR3 increases the production of IL-6, in addition to MIF (macrophage migration inhibitory factor), the main factor for leucocyte recruitment (Kota et al. 2014). Human nasal mucosa MSCs (NM-MSCs) induced a strong release of pro-inflammatory cytokines (IL-6 and IL-8) and type I interferon after

Fig. 1 (continued) adapter-inducing interferon-β, PI3K phosphoinositide 3-kinase, *AKT* protein kinase B, *GSK3β* glycogen synthase kinase 3 beta, *TRAF* TNF-receptor-associated factor, *RIP* receptor-interacting protein, *TAB* TGF-beta-activated kinase 1 (MAP3K7) binding protein, *TAK1* mitogen-activated protein kinase kinase kinase 7, *IKK* IκB kinase, *NF-κB* nuclear factor "kappa-light-chain-enhancer" of activated B cells, *IRF* interferon regulatory factor, *LEF* T cell factor/lymphoid enhancer-binding factor, *p38* p38 mitogen-activated protein kinase, *JNK* c-Jun N-terminal kinase, *AP-1* activator protein 1, *EBP* EBP cholestenol delta isomerase, and *MMK* mitogen-activated protein kinase kinase kinase

activation of TLR3 (Dumitru et al. 2014). Furthermore, investigations have reported the solid immune cell chemoattractant profile secreting CXCL1, CXCL5, CXCL6, CXCL8, and CXCL10 in TLR3-activated mesenchymal stromal cells from human tonsils (T-MSCs) (Ryu et al. 2015). Indeed, TLR3 pre-activation with Poly (I: C) in BM-MSCs also significantly increases the number of leukocytes binding to MSCs via interacting with hyaluronic acid structures (Kota et al. 2014).

After recruiting the immune cells to the MSCs surrounding area, diverse controlling mechanisms can participate in the progression of immune regulation. These mechanisms are complex but subtle to the microenvironment. In previous investigations, the results about TLR3 ligation and immunomodulation in this phase are contradictory and reported differently.

Waterman and colleagues have described a new paradigm for MSCs immunomodulation following TLR3 priming (Waterman et al. 2010). TLR3 priming shifted the MSCs into a particular phenotype (MSC2), mainly expressing immunosuppressive mediators as TGF-β, IDO (indoleamine-2,3-dioxygenase), PGE2 (prostaglandin E2), NO (nitric oxide), hemoxygenase (HO), and hepatocyte growth factor (HGF), leading to T cell inhibition. Similarly, other investigators described the TLR3-induced enhancement of immunosuppressive properties in BM-MSCs (Opitz et al. 2009) and G-MSCs (Mekhemar et al. 2018a) in response to the increased production of IDO1, as well as by an amplified expression of cyclooxygenase-2 (COX-2) in UCB-MSCs (Zhao et al. 2014) and by modulating the TGF-β and IL-6 production in the dental pulp (DP-MSCs) and dental follicle (DF-MSCs) (Tomic et al. 2011). On the other hand, TLR3 ligation was described to differentially affect the inhibitory functions of BM-derived MSCs, WJ-derived MSCs, and adipose-tissue-derived mesenchymal stem cells (AT-MSCs) (Raicevic et al. 2011). Notably, the immunosuppressive potential of WJ-MSCs and AT-MSCs remained unaltered, while BM-MSCs showed decreased potential to suppress lymphocyte activation. Another study showed that priming of TLR3 significantly reduced BM-MSCs' ability to inhibit T cells proliferation (Liotta et al. 2008b). This effect was not associated with IDO and PGE2 pathway regulation, instead it involved the downregulation of Jagged-1 induced by TLR3 priming.

It has been previously emphasized that MSCs-facilitated T cell inhibition arises via the production of galectins. One of the published studies has confirmed that galectin-9, which BM-MSCs do not constitutively produce, is significantly induced upon TLR3-MSC interaction and is imperative for the immunosuppressive properties of MSCs (Gieseke et al. 2013). Additionally, stimulation of TLR3 improved the suppressive effect of T-MSCs against Th17 differentiation by augmenting the expression of programmed death ligand-1 (PD-L1) (Cho et al. 2017). TLR3-mediated preconditioning of the cells by Poly (I: C) also confirmed an enhanced efficacy of UC-MSCs through the TLR3-Jagged-1-Notch-1 pathway (Qiu et al. 2017). Following PGE2 and Jagged-1 upregulation, PGE2 then amplifies IL-10 production and permits the Treg cell differentiation. Furthermore, TLR3 ligation amplified IL-12p35, IL-6, IL-23p19, and IL-27p28 transcription in BM-MSCs (Raicevic et al. 2010). These IL-12-related cytokine members can initiate $CD4^+$ T

helper 1 (T_H1) differentiation and support a T-cell-mediated immune reaction and induce pro-inflammatory changes in BM-MSCs.

MSCs can also have immune-regulatory effects on cells of the innate immune branch. As stated in numerous published studies, TLR3 triggered BM-MSCs to support the function and survival of neutrophils (PMN) by enhancing the respiratory burst ability, CD11b expression, and the antiapoptotic effect in PMN (Cassatella et al. 2011). The biological properties regulated in PMN via TLR3-triggered BM-MSCs were facilitated by the combined function of IL-6, IFN-β, and GM-CS. TLR3-primed MSCs also showed enhanced suppressive functions against NK cells (Noronha et al. 2019).

It seems that TLR3 and its ligands can assist as controllers of MSCs' immunomodulatory functions. Still, the effects are conflicting and possibly dependent on investigational settings and the microenvironment of the cells.

TLR3 Priming and MSC Differentiation

MSCs' capacity for multilineage differentiation potential to adopt diverging tissue phenotypes has been often explained as an age-dependent process (Sallustio et al. 2019). Recent investigations underline the important functions displayed by TLR ligands in guiding MSCs' maturation to form various cell phenotypes and in fate determination. The stimulation of TLR3 has been presented to affect osteogenic differentiation of MSCs and their maturation into osteocytes (Liotta et al. 2008b; Lombardo et al. 2009b; Pevsner-Fischer et al. 2007; Mekhemar et al. 2020). TLR-3 ligation via Poly (I: C) was described to endorse osteogenesis by supporting the BM-MSCs' differentiation into osteoblasts (Qi et al. 2014). Similarly, another investigation (Lombardo et al. 2009b) has demonstrated that TLR3 Poly (I: C) activation of AT-MSCs augmented their osteogenic differentiation potential (Hwa et al. 2006). Poly (I: C)-activated G-MSCs showed a lower expression of ALP and RUNX as early osteogenic markers with a concomitantly increased mineral deposition observed by the alizarin red staining (Mekhemar et al. 2020). Synchronized to this effect on MSCs bone maturation, an interplay between downregulation of pluripotency factors, as well as proliferation potential, and colony-forming units of TLR3-ligated G-MSCs have been observed presenting a cell cycle shift from pluripotency to the stages of differentiation and maturation (Mekhemar et al. 2020). This modulatory process of differentiation and cell-fate determination through TLR3 activation in MSCs was explained to be involved via upregulated β-catenin with regulatory NF-κB co-activation (Jia et al. 2015; Katoh and Katoh 2007; Ma and Hottiger 2016; Park et al. 2016; Yeh et al. 2016) and further facilitated by phosphorylation of the AKT pathway, which contributes greatly to MSCs' osteogenic differentiation and maturation (Baker et al. 2015; Joung et al. 2011; Qi et al. 2014; Zhu et al. 2019). However, some studies have reported that the activation of TLR3 inhibited the differentiation of UC-MSCs into osteocytes (Zhang et al. 2015) or did not affect the differentiation potential of BM-MSCs (Liotta et al. 2008a). Although TLR3 has shown a similar supporting role in promoting

adipogenic and chondrogenic maturation of MSCs in some investigations so far, opposing results occur regarding the effect of TLR3 on MSCs' maturation into these lineages (Mekhemar et al. 2020). As reported by multiple studies, TLR3 ligation outlined a fragile adipogenic modulation of MSCs, mediated by the phosphorylation of β-catenin and GSK3β inhibition leading to a partly reverse arrangement between the induction of MSC osteogenic and adipogenic differentiation (Mekhemar et al. 2020; Hwang et al. 2014; Lombardo et al. 2009a). Furthermore, additional studies clarified the stimulatory effect of β-catenin pathways in response to TLR3 stimulation on MSCs. They showed increased chondrogenic and osteogenic differentiation of MSCs with an attenuated adipogenic lineage effect (Mekhemar et al. 2020; Galli et al. 2013; James 2013; Li et al. 2008). It appears that TLR3 activation can function as a modulator of differentiation potential and may determine the cell fate besides maintaining their multipotency.

TLR3 Priming and MSCs' Migration and Proliferation

Continued growth and maintenance of MSCs are important characteristics much needed for their therapeutic effect as they eventually endure replicative senescence after prolonged periods of normal growth (Ciria et al. 2017). MSCs possess the significant ability to relocate to the areas of the inflammatory response, ischemia, and mechanical damage or even to sites of growing tumors (Kholodenko et al. 2013). TLRs have been suggested to moderate MSCs' proliferation, immunomodulation, as well as migratory activity, and differentiation potential (Sallustio et al. 2019).

Various studies have shown that TLR3 is a key mediator in migratory responses of BM-MSCs (Tomchuck et al. 2008). Still, this consequence seems to be connected to the exposure time: After one-hour incubation, TLR3 activation promoted migration, while after 24 h of incubation, the rate of migration and invasion of the treated MSCs became suppressed (Liotta et al. 2008b; Waterman et al. 2010). Furthermore, abrogation of TLR3 expression using knockdown plasmids reduced the migration potential of the nonactivated MSCs to the half (Tomchuck et al. 2008). Nonetheless, Poly (I: C) ligation of the transfected cells caused an improved migration potential when associated with unligated controls (Waterman et al. 2010). These different reactions of MSCs migration, reliant on marginal changes in the cell setting, support the TLR3 regulation of MSCs migration by multifaceted and generally unraveled molecular processes.

On the level of TLR3-mediated MSCs proliferation, TLR3 activation and expression was linked to suppressed proliferation in neuronal stem/progenitor cells (NPCs) (Sallustio et al. 2019; Lathia et al. 2008) and UCB-MSCs (Sallustio et al. 2019). Correspondingly, TLR3-activated G-MSCs displayed a decreased proliferative potential (Mekhemar et al. 2020), resembling a prior designated antiproliferative effect of Poly (I: C) on BM-MSCs (Choumerianou et al. 2010). As the TLR3 pathway begins with TRIF involvement, the IRF3 pathway consequently gets triggered, leading to cell proliferation inhibition (Dumitru et al. 2014; Lathia et al. 2008; Mekhemar et al. 2020). Additionally, the TRIF-independent branch of TLR3

signaling may similarly add to the suppression of cell proliferation via the c-Src pathway (Yamashita et al. 2012b).

TLR3-Mediated Apoptosis and Survival of MSCs

In cancer research, TLR3 ligands have become promising pharmaceuticals in immune-based chemotherapy. Investigations dealing with different tumor entities revealed direct apoptotic induction after TLR3 activation (Otkur et al. 2018; Zhao et al. 2012). Similar effects have been reported in fibroblast-like synoviocytes (Karpus et al. 2016). This direct induction is mainly based on TLR3-dependent TRAF6 and NF-κB activation, leading to INF-induced apoptosis (Chiron et al. 2009). Similar effects have been shown in adipose cells, which make TLR3 an attractive target in chronic fatigue treatment (Strayer et al. 2012). Besides its direct influences, TLR3 provides immunomodulatory effects on NK-cells as key players during innate immune response (Petri et al. 2017). Remarkably, Giuliani et al. (2014) showed protective effects of TLR3 primed MSCs against NK-cell killing (Giuliani et al. 2014).

TLR3 stimulation also increases the therapeutic potential of MSCs and causes 40% less apoptosis and improved regeneration in heart failure models (Mastri et al. 2012; Shabbir et al. 2009). However, the effect of TLR3 activation on MSCs under pro-inflammatory and regeneration conditions have not been completely unveiled yet and offer promising targets in tissue regeneration studies in future.

Micro-RNAs as Modulators of TLR3-Mediated Response in MSCs

Micro-RNAs (miRNAs) comprise noncoding RNA regulating gene expression by coupling with messenger RNAs (mRNAs) and inhibiting their translation to form proteins. These minor RNA molecules are mainly transcribed inside the cell nucleus and transformed into originator miRNA and then conveyed to the cytosol to be altered to developed miRNA by Argonaut and DICER proteins (Gulyaeva and Kushlinskiy 2016). Every sequence of miRNA can bind to and inhibit the expression of multiple mRNAs (Avraham and Yarden 2012), making them greatly important in various cellular functions and hence for the study of human cellular disorders and their treatments (O'connell et al. 2012).

Given MSCs' extensive and critical involvement in diverse cellular functions, they are being induced by preconditioning of stem cells (Suzuki et al. 2010) or through genetic manipulation with the miRNA of interest to enhance their functionality (Kim et al. 2012a, b) and reparability (Haider and Aramini 2019). Pharmacological preconditioning has also been shown to enhance their survival and angiomyogenic potential via phosphorylation of PI3K, AKt, GSK3b, and nuclear NFkB (Afzal et al. 2010). By interfering with the cellular functions in the immune cells and their precursors, miRNAs regulate numerous functional features of innate and acquired immune reactions. In pre-T and pre-B lymphocytes, inhibition of

DICER protein revokes differentiation and growth of these cells (O'connell et al. 2012). miRNAs can moderate the role of immune cells by directing diverse intracellular signaling pathways, i.e., the Wnt pathway in dendritic cells (Luo et al. 2015), as well as the Akt signaling in B lymphocytes and the TLR pathways (He et al. 2013; O'connell et al. 2007; Fehniger 2013). Recently, many studies have recognized a collection of miRNAs that control the characteristics of MSCs, as multilineage differentiation and immunomodulation (Clark et al. 2014). These miRNAs can impact the communication between MSCs and the cells of the immune system by affecting the mechanisms involving TLR signaling pathways, as several molecules in TLR signaling, their related cytokines, and transcription factors are involving in there (Clark et al. 2014). Therefore, the molecular exchange between miRNAs and the TLR pathways in MSCs may promote inhibition or stimulation of the TLR signaling pathway and responses in MSCs (Abdi et al. 2018).

For example, miR-143 has been reported as a critical regulator of MSCs' cell cycle activity via transcriptional regulation of cyclin D (Lai et al. 2011, 2012). In MSCs-TLR3-mediated signaling, miR-143 plays an effective role as a negative regulator. In human umbilical cord MSCs, TLR3 ligands significantly amplified the secretion of cytokines and chemokines as COX2, IDO, and PGE2, IL-6, and IL-8, which was linked to a miR-143 downregulation (Abdi et al. 2018). When upregulated, miR-143 abrogated cytokine production and reduced the immunosuppressive effects of TLR3-activated MSCs, besides reducing the beneficial properties of MSCs in an experimental animal model of sepsis (Zhao et al. 2014). In these studies, COX2 and TAK1 genes were directly targeted and downregulated by miR-143. These data show that TLR3 stimulation moderates immune effects of UCB-MSCs through inhibition of miR-143. Nevertheless, more future investigations are required to determine the modulatory role of miRNAs in MSCs involved in cell-fate determination and regulatory functions of immune reactions including T cell proliferation and macrophage polarization in the context of TLR3 activation.

Conclusion

Exploring TLR-mediated effects on MSCs' immunobiological and regenerative functions remains an area of immense importance to facilitate effective therapeutic utilization of MSCs. In this chapter, we have discussed the involvement of TLR3 in regulating diverse MSC functions. The listed studies in the chapter provide strong evidence that TLR3 priming could critically impact the cell-fate determination, multilineage potential, hematopoietic support, immunomodulatory ability, as well as the apoptosis, migration, and proliferation of MSCs from different tissue sources as main thus impacting the outcome of various MSCs-based therapies. Consequently, preconditioning of MSCs to be employed clinically may be treated with TLR3 ligands to enhance their therapeutic effect post-engraftment (Delarosa et al. 2009). In this regard, TLR3 stimulating agents are also being studied as potential vaccine adjuvants for cancers, infectious diseases (Seya et al. 2015; Lebedeva et al. 2018), or as a highly specific novel treatment strategy against tumors (Le Naour et al. 2020).

Likewise, inhibitors of TLR3 signaling have shown considerable potential in the treatment of inflammatory disorders (Dunne et al. 2011). Notwithstanding a large amount of information gained about TLR3-mediated effects on MSCs, the data emanating from these studies remain inconsistent. These discrepancies and inconsistencies observed in the published data have been attributed to the multiplicity of experimental settings and protocols employed to learn the impact of TLR3 activity level on MSCs. Notably, the influence of tissue source, culture conditions, purity level, etc., may play an essential role determining the outcome of the studies. In this context, we need to well design and standardize the investigations to better understand the mechanism of action of TLR3 on MSCs and precisely acquire the needed information to control TLR3 and its downstream pathways to modulate the needed MSCs characteristic features in the pathological environment. These data would certainly help in improving the safety and therapeutic potential of MSCs-based therapy in the future.

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Targeting Cancer Stem Cells: New Perspectives for a Cure to Cancer

44

Beatrice Aramini, Valentina Masciale, Giulia Grisendi, Federico Banchelli, Roberto D'Amico, Massimo Dominici, and Khawaja Husnain Haider

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B. Aramini (✉)
Division of Thoracic Surgery, Department of Experimental, Diagnostic and Specialty Medicine–DIMES of the Alma Mater Studiorum, University of Bologna, G.B. Morgagni–L. Pierantoni Hospital, Forlì, Italy
e-mail: beatrice.aramini2@unibo.it

V. Masciale
Division of Thoracic Surgery, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy
e-mail: valentina.masciale@unimore.it

G. Grisendi · M. Dominici
Division of Oncology, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy
e-mail: giulia.grisendi@unimore.it; massimo.dominici@unimore.it

F. Banchelli · R. D'Amico
Center of Statistic, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy
e-mail: roberto.damico@unimore.i

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

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Abstract

Cancer stem cells (CSCs) are present in many solid tumors. Their signaling pathways and functions may be the key to developing new anticancer strategies against cancer. Several studies have linked the signaling pathways' (e.g., Wnt, Notch, and Hedgehog pathways) aberrant activation to the development of numerous cancers. These signaling pathways hence provide attractive targets for further study toward new therapies. CSCs show several characteristics, such as self-renewal, differentiation, high tumorigenicity, and resistance to anticancer drugs. Drug resistance is the most useful in further evaluating the possibilities of reducing tumor mass or eliminating cancer by discovering new therapies. One of the key questions concerns the necessity of identifying superficial as well as intracellular markers, which are essential if the drug is to respond positively to CSCs. In recent years, CD133, CD44, ABCG2, and ALDH have been identified as biomarkers for certain forms of CSCs. However, recent studies have contributed to a better understanding of CSC-specific antigens; to date, there is no univocal characterization of antigens for CSCs. Our chapter aims to highlight the importance of identifying new markers to develop new therapeutic strategies against cancer.

Keywords

Cancer · Cancer stem cells · Chemotherapy · Radioresistance · Tumor

List of Abbreviations

ABCG2	ATP-binding cassette subfamily-G member 2 (ABCG2)
ALDH1	Aldehyde dehydrogenase1
CSCs	Cancer stem cells
Gli	Glioma-associated oncogene homolog
GCSF	Granulocyte colony-stimulating factor
HCS	High-content screening
HDECC	High drug efflux cancer cell inhibitors
HMGA2	High mobility group AT-hook 2
IFN-α	Interferon-α
MDR	Multidrug-resistant proteins
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

Introduction

Role of Cancer Stem Cells (CSCs) in the Tumor

One of the recurring problems that remain unresolved about cancer is the possibility of developing local and/or distant recurrence (Sopik et al. 2018; Hung et al. 2012). The tumor relapse comes from its multiple cell types' composition, which contributes to our capacity to abolish tumor to replication. CSCs partially control the cancer heterogeneity CSCs described for the first time in 1928 by Krebs and colleagues. They discovered similarities in the mechanisms involved in tumor development and the development of embryos (Krebs 1947). However, the first real CSC model was reported in a small population of cells considered *the leukemia promoter* (Terstappen et al. 1991; Bao et al. 2013; Schulenburg et al. 2015). The subsequent studies have shown that the only cell type capable of forming tumors was CSCs. This aspect was demonstrated in different types of cancers when CSCs were implanted into experimental animal models. Only in 2005, the scientists were able to test the presence of these tumor-initiating cells in solid cancers, such as in breast, brain, and intestine cancer, and melanoma using a transgenic mouse model (Cho et al. 2008; Alcantara Llaguno et al. 2009; Barker et al. 2009; Fang et al. 2005).

CSCs have been extensively studied and characterized during the last decade. CSCs show similar characteristics to normal stem cells, such as the capacity to grow, self-renew, and disseminate to other tissues and organs during the metastatic process (Bonnet and Dick 1997). CSCs show resistance to apoptosis besides possessing the ability to escape immune surveillance. This involvement of CSCs and lymphocytes has been already discussed in the recently published literature (Ravindran et al. 2019). In a study published by Masciale et al. (2019a), the authors have reported a correlation between cytotoxic tumor-infiltrating lymphocytes CD3+ and CD8+ and lung CSCs, which could be useful for the development of future therapies in the field of precision medicine, tailored on the specific marker besides the different roles of lymphocytes against lung cancer. These data show that lymphocytes may have a strategic role in tumor development and cell-cell interactions.

In summary, CSCs interact with tumor epithelial cells, tumor microenvironment, and tumor-infiltrating lymphocytes. They are considered as one of the most important causes of resistance to chemotherapy besides their involvement in radioresistance and loss of the DNA repair of the tumor cells inside the tumor caused by radiations. These also induce the destruction of mitochondrial membranes and functions by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Schulz et al. 2019; Pilié et al. 2019; Azzam et al. 2012; Schieber and Chandel 2014). The physiologic intracellular mechanisms can maintain ROS' presence, which may protect the cells against the oxidative stress induced by radiations (Trachootham et al. 2009).

Several studies have attempted to identify the leading causes of resistance due to CSCs. They found that many intracellular pathologic factors such as quiescence, dormancy, transporter accumulation, cell reprogramming, interruption of apoptosis, and metabolic pathways may reinforce the tumor resistance to chemotherapy and radiotherapy of this subpopulation of cells. The commonly used treatment for

oncologic patients, including surgical resection, may reduce the tumor mass but fails to destroy all the cells with the cancer relapse (Arruebo et al. 2011). For this reason, scientists believe that the development of new targeted drugs able to target CSC will be beneficial to overcome tumor resistance. The possibility of CSC-targeted treatment options will provide a novel "door opening door" for the development of more personalized medicine in the field of solid tumors.

Clinical trials are underway to study the pharmacokinetics, safety, and efficacy of combination treatment; however, there are no drugs available for lung cancer that specifically could target CSCs (Aramini et al. 2020a). The combination of "old treatments" associated with targeted CSC-specific agents will probably set a more personalized oncologic medicine that may be more promising in terms of response than the existing anticancer therapies.

Markers in CSCS: A New Approach Against Tumor

One of the most debated aspects of CSCs is their existence and identification of tumors (Prager et al. 2019). The scientists have attempted to characterize several solid tumors for the presence of CSCs for their surface markers, although the discussion is still open for the complexity of their identification (Sun et al. 2019). Identifying CSCs in human tissue or cell lines is essential to set effective new target therapies against cancer. As CSCs have many characteristics in common with normal stem cells, the scientists believe that specific markers, as superficial markers, are especially useful to identify them (Franco et al. 2016).

For their innate characteristics, as the capacity to form non-adherent spheres and their intracellular mechanisms, several assays have been found to identify CSCs (Dou and Gu 2010; Alison et al. 2011; Ghani et al. 2011; Zhang et al. 2011; Dou et al. 2007). In particular, in vitro studies demonstrated that markers as aldehyde dehydrogenase1 (ALDH1), CD133, and others might be suitable for identifying CSCs (Zhao et al. 2017; Kim and Ryu 2017; Klonisch et al. 2008). Besides surface markers, specific signaling pathways, sphere-forming assays, and other relevant specific assays are being developed for CSC identification and characterization (Table 1). However, to demonstrate CSCs' presence, in vivo experiments are being used to augment previously reported data from the in vitro studies. These data are significant to characterize CSCs in terms of tumor development and dissemination. For this reason, scientists have opted to transplant CSCs into experimental animal models. Despite the importance of these models, which are in use since 1902 for genetic studies on mice (Cheng et al. 2010; Aiken and Werbowetski-Ogilvie 2013), new technologies have emerged during the last decade. For example, 3D culture technologies, which aid preclinical studies for drug testing and promote the tumor-sphere formation, represent the first step in simulating the in vivo model (Ericsson et al. 2013; Zhang et al. 2020; Candini et al. 2020). The determination of this malignant cell subpopulation, CSCs, in the tumor mass remains a complicated process. Surface markers are the most commonly and frequently used for identifying CSCs in many solid tumors (Xia 2014; Sullivan et al. 2010).

Table 1 Therapeutic attempts to target CSC

Target	Cancer type	Effector	Preclinical result	References
ALDH	Melanoma	Nanodisc (ND) vaccination	Nanodisc vaccination against $ALDH^{high}$ CSCs combined with anti-PD-L1 therapy exerted potent antitumor efficacy and prolonged animal survival in multiple murine mode	Hassani et al. (2020)
ALDH	NSCLC	DIMATE, an irreversible inhibitor of ALDH1/3	In lung cancer xenografts with high to moderate cisplatin resistance, combination treatment with DIMATE promoted strong synergistic responses with tumor regression	Rebollido-Rios et al. (2020)
ALDH1A1	Ovarian cancer	Solanum incanum extract	Notch1 and FoxM1 were downregulated, which resulted in increased chemotherapeutic sensitivities	Wu et al. (2015)
ALDH	Ovarian cancer	Disulfiram (DSF)	DSF enhances cisplatin cytotoxicity in ALDH + cells	Guo et al. (2019)
STAT3	Gastro/intestinal cancer	Napabucasin (BBI-608, Boston Biomedical Inc., MA, USA)	BBI-608 target and inhibit gene transcription induced by STAT3 and cancer cell stemness properties	Sonbol et al. (2019)
CD133	Hepatocellular carcinoma, colorectal cancer, and melanoma	SS2 virus, an oncolytic HSV-1	SS2 inhibits in vivo growth of tumors in subcutaneous mouse model for melanoma, hepatocellular carcinoma, and colorectal cancer	Terai et al. (2018)
CD133	Testicular and embryonal carcinoma	BST204-a fermented ginseng extract	Treatment with BST204 (25, 50, and 100 μg/mL) inhibited the proliferation of NCCIT cells in a dose-dependent manner	Park et al. (2020)
Hedgehog	Bladder	Cyclopamine	Tumor formation was suppressed via inhibition of GALNT1 that mediates SHH signaling	Li et al. (2016)
Hedgehog	NSCLC and small-cell lung cancer	GDC-0449	GDC-0449, an inhibitor of the hedgehog pathway, demonstrated its efficacy in both NSCLC and small-cell lung cancer via suppression of stemness-related features	Shibata and Hoque (2019)

(continued)

Table 1 (continued)

Target	Cancer type	Effector	Preclinical result	References
Hedgehog	Ovarian cancer	GDC-0449 (vismodegib derivative) and sonidegib (LDE225)	Since the SHH pathway plays an important role in self-implantation of CSC and other characteristics of these cells, its inhibition may disrupt CSC stemness through differentiation of these cells	Keyvani et al. (2019)
KLF5	Breast carcinoma	Metformin	Metformin suppresses TNBC stem cells partially through the inhibition of KLF5	Shi et al. (2017)
KLF4/PI3K/Akt/p21	Prostate cancer	MicroRNA-7	miR-7 is as a suppressor of prostate CSCs and via suppressing the KLF4/PI3K/Akt/p21 pathway	Chang et al. (2015)
Wnt/β-catenin	Breast cancer	Pyrvinium pamoate	$CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ cells were suppressed by downregulating *NANOG*, *OCT4*, and *SOX2*	Rodriguez et al. (2019)
Wnt/β-catenin	Breast cancer	Resveratrol	Resveratrol could eliminate breast CSCs in primary xenografts and consequently abrogate the regrowth of tumors in secondary mice	Fu et al. (2014)
YAP1	Head and neck squamous carcinoma cells	Verteporfin	Verteporfin suppresses the proliferation, epithelial-mesenchymal transition, and stemness of head and neck squamous carcinoma cells	Liu et al. (2019)

The most established surface markers to identify CSCs are CD133, CD24, and CD44 besides intracellular enzyme aldehyde dehydrogenase (ALDH), which relates well to both solid tumors and lung cancer (Qiu et al. 2019). The main issue regarding surface markers is to discriminate between their isoform expressions in various organs. For example, Al-Hajj et al. found that CD44/CD24 surface marker expression was related to the cells' capacity to form a tumor mass, while its other isoforms did not show this potential (Al-Hajj et al. 2003). Similarly, Li et al. described similar results have for other organ cancers, i.e., in human pancreatic cancer (Li et al. 2007). Regarding lung cancer, for example, Sullivan et al. 2010 tested ALDH in several cell lines and human tissue samples (Sullivan et al. 2010). In 2019, Masciale et al. compared the isolated $ALDH^{high}$ population with the marker CD44+. They found that these two subpopulations did not match exactly and found a broader population

in CD44+ than in ALDH^{high} (Masciale et al. 2019, 2020b). The comparison between ALDH^{high} and CD44+/EpCAM+ identified a remarkably similar cell population. This study represents the first attempt demonstrating how markers that seemingly identify CSCs do not always identify similar populations. In summary, the consideration of surface markers is not enough to isolate CSCs and, therefore, necessitate combining surface markers with other mechanisms to identify and target them.

Targeting CSCs

The Hedgehog Signaling Pathway

CSC formation seems to primarily derive from the dysregulation or the aberrant activation of intracellular signaling pathways which are numerous (Matsui 2016). The most well studied of these signaling pathways include Hedgehog pathway, Notch, Wnt/b-catenin, the high mobility group AT-hook 2 (HMGA2), Bcl-2, and Bmi-1, among many others (Pelullo et al. 2019). Further in-depth knowledge of these mechanisms will allow the researchers to exploit them to find novel anticancer therapies. Signaling pathways generally comprise the expression of a network of genes and their encoded proteins that can regulate diverse intracellular processes. These pathways are significantly involved in self-renewal, maintenance, proliferation, differentiation, and cell migration. In contrast, the aberrant expression of the genes/ protein involved therein or their dysregulation can lead to the development of CSCs, which are the inductors of tumorigenesis (Olivares-Urbano et al. 2020).

For example, the Hedgehog signaling pathway controls several biological activities in the normal cells and stem cells' development, and it is central to the regulation of different solid tumors (Olivares-Urbano et al. 2020; Petrova and Joyner 2014). Hedgehog signaling's dysregulated activation signaling is crucial for tumor initiation, infiltration, dissemination, and chemoresistance. In particular, the activation of Hedgehog signaling is initiated by the interaction between the Hedgehog protein and the cellular transmembrane patched (Ptch), resulting in a gene cascade control that is crucial for several cell functions as the cells proliferate, disseminate, and self-renew (Jia et al. 2015). The Hh signaling's abnormal activation induces cellular alteration processes with the consequent transformation of normal cells into cancer cells. In particular, recently published data show that Hedgehog signaling forms the basis of CSC equilibrium in many solid tumors (Pietrobono et al. 2019; Gotschel et al. 2013; Filbin et al. 2013; Gulino et al. 2010), and hence it seems to be one of the leading causes of resistance against cancer therapy (Gurung et al. 2013).

Recent studies have studied the effect of inhibiting the Hedgehog signaling pathway (Pietrobono et al. 2019; Gotschel et al. 2013; Filbin et al. 2013; Gulino et al. 2010; Gupta et al. 2010). For example, the drugs, which inhibit the molecule smoothened (SMO), i.e., cyclopamine and GDC-0449 (vismodegib), do not perform well against cancers harboring lesions that lie downstream of the SMO (Gupta et al. 2010). Other drugs seem to be effective against the glioma-associated oncogene

homolog (Gli) proteins and the Gli transcriptional activity or the combination of different factors with the inhibition of CSCs' main characteristics, for example, the capacity to self-generate (Gurung et al. 2013).

The Notch Signaling Pathway

The Notch pathway has a vital role in the communications that occur among adult stem cells and during embryo development (Yu et al. 2008; Nguyen et al. 2006). Four receptors (Notch-1–Notch-4) and their ligands Deltalike1, Deltalike3, and Deltalike4 and Jagged 1 and Jagged 2 activate the Notch with a cascade of multiple genes (Serneels et al. 2005; D'Souza et al. 2010; Kovall et al. 2017). The Notch pathway's prominent role is in stem cell evolution, from initiation to apoptosis, but it also has an oncogenic/oncosuppressive role, depending on the tumor microenvironment (Nowell and Radtke 2013). Besides its pivotal role in intercellular communication, Notch signaling's most interesting characteristic is its double function as an oncogenic protein in several solid tumors. In contrast, it acts as a tumor suppressor in others (Dotto 2008). Given this differential role, researchers have started to consider it a potential novel target to eliminate CSCs (Wang et al. 2010, 2011; Pannuti et al. 2010; Qiao and Wong 2009; Fan et al. 2010). Tetering et al. have reported that abrogation of the Notch pathway blocks the proteolytic process (Van Tetering and Vooijs 2011). Lim et al. used g-secretase inhibitors (GSIs) to reduce primary stem cell characteristics (Lim et al. 2015). They asserted that blocking the Notch pathway in glioblastoma helps establish new target therapies to counter CSCs. Treatment with GSI MRK-003 destroys cancer stemlike cells, with the consequent blocking of the tumor and dissemination into the body (Venkatesh et al. 2018; Grudzien et al. 2010; Ng et al. 2019). The only limitation is that GSI MRK-003 is nonselective as it could block four Notch ligands and several substrates of the g-secretase. Despite interesting data, further mechanistic investigations are required to understand its toxic effects on human organs.

The Wnt Signaling Pathway

Another critical pathway subject of recent interest and useful for developing future generations' targeted therapies against CSCs is the Wnt signaling pathway, which is involved in all the steps about the stem cell generation, progression, up to differentiation (Ng et al. 2019; Komiya and Habas 2008). The most rigorously defined Wnt pathways are the Wnt/b-catenin signaling pathway that starts when a Wnt glycoprotein binds with a cell receptor to initiate the activation of a cascade (Macdonald et al. 2009; Franch-Marro et al. 2008). The role of the Wnt pathway is crucial for stem cell regeneration and the preservation of CSCs; however, mutations in the signaling molecules or inactivation of cell processes may induce the dysregulation of this pathway with the induction of cell proliferation and tumor dissemination (Huang and He 2008; Itasaki et al. 2003; Itoh et al. 2005).

Nevertheless, mutations can interfere not only with the Wnt signaling pathway but also with the tumor microenvironment. For example, biologic inhibitors or drugs may block or prevent the interactions between the ligand and the receptor. These small molecules can inhibit the Wnt target enzymes, reduce CSCs' drug resistance and proliferation, etc. (Jamora et al. 2003; Artem et al. 2020). A study published in 2017 by Wiese et al. (2017) showed that the drug ICG-001 might positively effect tumorigenicity of pediatric glioma tumorigenicity by a Wnt-independent regulation of genes. Another technique used for inhibiting Wnt signaling is the RNA interference technology to downregulate the beta-catenin expression, resultant reduction in several CSC properties, and the silencing of stemness genes as OCT-4 (Hadjimichael et al. 2015).

In summary, targeting the Wnt pathway is another practical and effective approach in developing and investigating the future of targeted treatment approach for solid tumors. This capacity to target this mechanism will also be essential to better control normal stem cell activities, differentiating CSCs from normal ones in tumors (Takahashi-Yanaga and Kahn 2010; Yang et al. 2020; Vassalli 2019). This aspect would help target only the cancerous cells in the tumor.

Targeting ALDH as the Drug-Detoxify Enzymes Against CSCs

ALDH is the most used marker for the identification of CSCs. During these past 10 years, several publications described its role as a useful and reliable predictive marker for CSCs (Masciale et al. 2019, 2020; Vassalli 2019; Ho and Weiner 2005; Walcher et al. 2020; Croker et al. 2009; https://clinicaltrials.gov/; Singh et al. 2013; Croker et al. 2017). In particular, in 2010, Sullivan et al. showed that ALDH-positive cells had the characteristics of cancer stemlike cells in NSCLC lines (Sullivan et al. 2010) and lung primary cell cultures. ALDH has also been described in benign diseases (Ho and Weiner 2005) although it is highly expressed in lung epithelial cancer cells. This aspect is exciting and opened the door of recent studies on ALDH as a NAD(P)þ-dependent enzyme catalyzing the aldehydes' oxidation into carboxylic acids (Ho and Weiner 2005). ALDH superfamily encompasses different isoenzymes, but the most used in detecting CSCs are ALDH1A1 and ALDH1A3. However, the possibility of targeting to set personalized therapy for CSCs is still debated (Walcher et al. 2020). For example, Masciale V. et al. recently demonstrated that the $ALDH^{high}$ cell population does not correlate with CD44+ cells in lung adenocarcinoma. However, the $ALDH^{high}$ cells are positively correlated with CD44+/EpCAM+ cell subpopulation (Masciale et al. 2020).

Croker et al. (2009) demonstrated a subpopulation of cells identified by the ALDH marker, among others, might be the driver of metastasis in breast cancer and the cause of resistance to traditional chemotherapies or radiotherapy in epithelial cancer cell lines. However, although some clinical trials on ALDH (disulfiram and DIMATE) are running at the moment trying to identify a drug which can be effective against solid tumors (Singh et al. 2013; Croker et al. 2017), there is not enough clarification about ALDH isoform markers in terms of their predictive value and

targeted therapy against cancer. Several future studies are needed to increase the knowledge about this important family of enzymes.

Targeting Drug Efflux Pumps

The efflux systems to get rid of the xenobiotic substances in the cells are involved in diverse CSCs' processes similar to their involvement in normal stem cells. Their presence protects the cells from xenobiotic molecules such as chemotherapeutic agents, toxins, and drugs in general. Many ABC transporters' family members mediate the efflux of cytotoxic chemotherapeutics and are called multidrug-resistant (MDR) proteins. MDR proteins are the ATP-binding cassette subfamily-G member 2 (ABCG2) described in breast cancer resistant cells (BCRP) involved in CSC compartments as some MDR transporters are much more prominent in CSCs than cancer cells and healthy cells (Prabavathy et al. 2018). ABCG2 overexpression maintains stem cell compartments by supporting their ability to counteract chemotherapeutic agents (Xiao et al. 2006). ABCG2 is a characteristic feature of CSCs akin to other non-CSCs with self-renewal, lineage capacity, tumorigenicity, and expression of stem cell-specific markers (Xiao et al. 2006).

To visualize and identify potent high drug efflux cancer cell inhibitors (HDECC), scientists applied an image-based high-content screening (HCS) starting from 1280 pharmacologically active molecules (Xia et al. 2010; Ansbro et al. 2013). This assay showed that these inhibitors could overcome multidrug resistance, increasing the chemotherapy's efficacy or reducing the tumorigenicity of cancer cells that might affect stemlike cancer cells. The ABCG2 seems to be fundamental in detecting the side population of various cancer types. It is essential to consider that these above-cited markers equally identify CSCs and normal healthy stem cells. Information should be taken into account during targeted therapy to find the best treatment, avoiding side effects (Wijaya et al. 2017). Therefore, the combined use of chemotherapy and inhibitors precisely targeting ABC drug transporters of CSCs may serve as an effective strategy for eradicating CSCs.

Targeting the CSC Niche and the Quiescent State

The stem cell niche provides an ideal tissue microenvironment conducive to the growth and maintenance of stemness (Morrison and Spradling 2008). Regarding this aspect, both CSCs and normal stem cells reside in their respective CSCs' niche or a stem cells' niche. The conducive niche microenvironment provides nourishment and adequate signals to regulate, first, self-renewal and, second, normal physiological processes such as those of inflammation, epithelial to mesenchymal transition (EMT), hypoxia, and angiogenesis (Morrison and Spradling 2008). In particular, stemness depends on extrinsic factors disposed of by the cancer microenvironment (Cabarcas et al. 2011), as happens in the colon CSC population, described by Vermeulen et al., in which the Wnt pathway regulates the colon CSCs (Vermeulen

et al. 2010). Moreover, it is a condition in which the brain tumor's microvasculature was crucial for niche and consequently for the support of CSCs (Seidel et al. 2010). For that reason, the vascular niches should be targeted for future therapeutic strategies to affect CSCs. Hypoxia has a crucial role in tumor growth since the hypoxic tumor microenvironment also sustains CSCs (Seidel et al. 2010).

Regarding hypoxia, antiangiogenic therapies promote hypoxia within the CSCs' niche, causing radioresistance to the CSCs, as occurring with the use of sunitinib and bevacizumab, as described by Conley and Wicha (2012). In this study, there was an intra-tumoral hypoxic environment created that leads to a stimulation of CSCs. Consequently, the study suggested that antiangiogenic treatments in combination with CSC-targeted drugs may be a better and more effective option. For example, it is essential to note the positive anticancer effects produced by the inhibitors for hypoxia-inducible factor1a (HIF-1a) that could serve as a basis for effective therapy (Zhong et al. 2000). Indeed, targeting the CSCs' niche in combination with chemotherapy will be a therapeutic intervention approach for eradicating these cells. This is fundamental for decision-making during cancer therapy because CSCs are resistant to traditional chemotherapy due to their quiescent state (Iwasaki and Suda 2009; Cheung and Rando 2013).

Current treatment strategies can inhibit tumor growth or DNA synthesis but are ineffective as a long-term response to cancer. Unlike other cancer cells, CSCs can remain resting, shielded from the external stimuli to prevent their replicative potential (He et al. 2017; Cheung and Rando 2013). CSCs are slow-cycling cells, a characteristic that helps them protect against chemoradiotherapy agents (Kusumbe and Bapat 2009). Therefore, strategies that induce CSCs to enter the cell cycle will enhance their susceptibility to chemotherapy (Plaks et al. 2015). Recent research has shown interferon-α (IFN-α), granulocyte colony-stimulating factor (G-CSF), or As_2O_3 is useful in this regard (Han et al. 2013). Therefore, the combination of IFN-α, G-CSF, or As_2O_3 with chemotherapeutic factors may effectively target the dormant CSCs. The future advances in anticancer treatment may be characterized by therapeutic methods that can target the stem cell niche, affecting the primary source of nourishment and intracellular signal for CSCs (Han et al. 2013).

An Update on the Most Used Target Therapies Against CSCs

One of the main areas necessitating innovation in cancer treatment is studying the mechanism of the development of resistance and relapse of the disease. This is generally considered a kind of failure in anticancer therapy development due to an apparent lack of specific targeting that directly attacks the tumor. During the past 10 years, stem cells have attracted interest in the scientific community for biological characteristics such as self-renewal, the capacity to migrate, and the modulation of cell interactions, among others. The scientists have mainly focused their attention on discovering new markers to better identify these cell populations (Plaks et al. 2015; Han et al. 2013; Xuan et al. 2019). Diverse approaches have been considered for

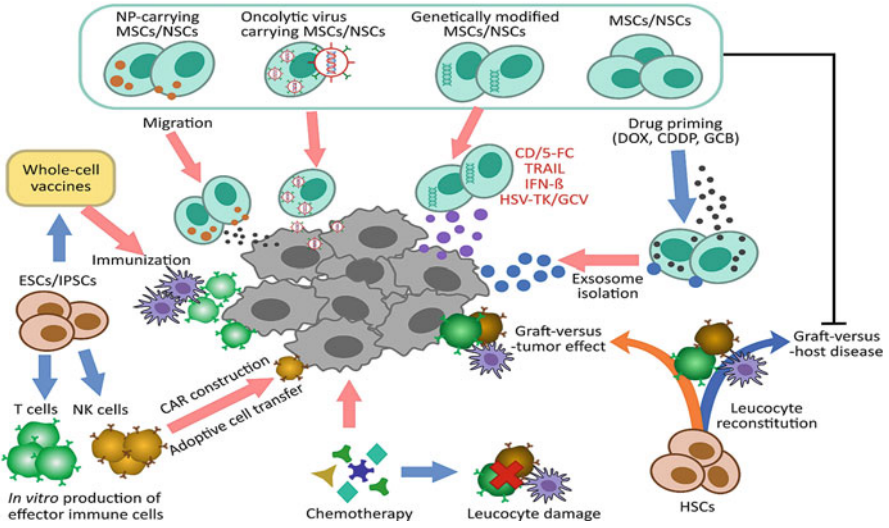

Fig. 1 Advanced cancer therapy. New perspectives in cancer therapies consist of different approaches using the great potential of the MSC by using genetic engineering, oncolytic virus, and nanoparticles releasing drugs or exosomes. Moreover, immunization technique with both vaccines and chimeric antibody is also investigated to enhance the immune response against cancer

stem cell therapy: HSC transplantation, MSC infusion, stem cells as carriers or generators of immune cell interactions, and vaccine production (Fig. 1).

In the last decade, biological characterization of CSCs has remained an area of immense interest, including their potential for self-renewal, the capacity to migrate, the modulation of cell interactions, etc. (Plaks et al. 2015; Han et al. 2013; Xuan et al. 2019). In particular, scientists have focused their attention on discovering new markers to identify these cell populations. There are ongoing difficulties in identifying unique markers. In particular, several vital drugs are not always effective in anticancer therapy, which is why patients have a frequent relapse and eventually succumb to the disease. One of the most urgent problems that require the immediate attention of researchers is drug resistance, which remains a challenge for modern medical oncologic treatments (Xuan et al. 2019). In particular, scientists discovered a small population of cells during the past two decades, similar to stem cells in their intrinsic characteristics but derived from the tumor itself. These cells are defined as CSCs and known for their self-renewal, differentiation, and tumorigenicity: these traits seem to be what causes cancer relapse and tumor dissemination (Han et al. 2013; Xuan et al. 2019; Kuczynski et al. 2019). Several interventional approaches have been tested to target this population of cells; however, currently, the two most utilized techniques in targeting CSCs are virotherapy through the oncolytic virus and miRNAs as CSC targets (Fig. 1).

An oncolytic virus (OV) destroys tumor cells with mechanisms that work differently from traditional medical treatments (Jain and Stylianopoulos 2010). These

mechanisms involve specific genes against cancer cells. However, when defining the possibilities and advantages of virotherapy, we should be aware and conscious of the limitations that hinder the development of new therapies for solid tumors. Scientists are attempting to exploit specific characteristics of the virus, i.e., tropism, the viral capacity of diffusion into the body, antiviral immunity, the delivery platforms, etc. (Maroun et al. 2017). However, the unique obstacles hindering their development include the physical barriers that prevent viral diffusion into the tumor and its passage through the endothelium (Kuczynski et al. 2019). A significant barrier for the OVs can be the host's immune surveillance mechanism. Bioactive factors, including cytokines and chemokines, and growth factors secreted by the tumor cells activate immune cells against OVs.

Additionally, the OVs may develop a robust innate response with an uncontrolled reaction against cancer but also against other tissues (Jain and Stylianopoulos 2010; Maroun et al. 2017; Hagedorn and Kreppel 2017; Guedan and Alemany 2018; Sharp and Lattime 2016; Nayyar et al. 2019; Oh et al. 2018; Vaupel 2004). It is pertinent to mention that OVs are capable of stimulating an effective immune response, both specific and innate, and the massive release of coagulation factors together with the complement proteins (Jain and Stylianopoulos 2010; Maroun et al. 2017; Hagedorn and Kreppel 2017; Guedan and Alemany 2018; Sharp and Lattime 2016; Nayyar et al. 2019; Oh et al. 2018; Vaupel 2004). It remains undetermined how many OVs reach the tumor-specific site, as they are very likely to be eliminated by the immune system (Hagedorn and Kreppel 2017; Guedan and Alemany 2018; Sharp and Lattime 2016). Once OVs target the tumor, the most crucial aspect is that they maintain intrinsic properties, such as the capacity to induce immune suppression of the TME to kill cancer cells and block tumor cell dissemination. Unfortunately, current studies are not setting out how to improve virotherapy's effectiveness against cancer in terms of tumor targeting, virus delivery, and dissemination. Therefore, it is imperative to distinguish the best virus species for OVs to reduce the events of tumor-targeting failures (Zheng et al. 2019).

Oncolytic Adenovirus

During the past two decades, oncolytic adenovirus (Ad) has gained popularity for cancer gene therapy primarily due to its ability to precisely infect and replicate in tumor cells. Given that the oncolytic antitumor activity is inadequate to eradicate tumors, different strategies based on genetic engineering have been developed to improve the efficacy of the cancer-targeting gene-virotherapy (Liu et al. 2012; Zhang et al. 2003). There are examples of genetically modified Ad eliminating E1B protein which blocks a p53-independent apoptosis mechanism or a portion of E1A involved in binding the retinoblastoma (Rb) protein since the significant part of CSCs has a defect in Rb and/or p53 pathway (Wang et al. 2015b; Lei et al. 2013). For this transcriptional target therapy, a specific cancer promoter has been used to control the

initial phases of the viral replication. Recently, GP73 protein and its promoter were used to regulate the activity of the E1A and E1B proteins to treat hepatocellular carcinoma (Mao et al. 2010; Fimmel and Wright 2009; Wang et al. 2015; Zhang et al. 2016). For example, the ZD55 and GD55 oncolytic viruses were tested for efficacy against lung CSCs. The result, much higher with GD55, was a significant increase in apoptosis rate due to the cytotoxic effect and oncolysis. Indeed, GD55 could represent a hopeful agent to develop target therapy for lung CSCs.

Regarding ZD55, as reported by Zhang and colleagues (2003), the increased acetylcholinesterase activity induced by the OV is responsible for a substantial antitumor effect against gastric cancer. This result is achieved by inhibiting the tumor growth in the gastric cancer cells and stem cells, whereas a low dosage of the ZD55 OV affects apoptosis (Zhang et al. 2003; Xu et al. 2014). Instead, Yano et al. investigated the efficacy of OB-301 telomerase-specific OV against gastric cancer cells (Yano et al. 2013). The oncolytic adenovirus allowed an efficient response by the killing of CD133+ cells responsible for chemoresistance and radioresistance. The CD133+ cells contributed to protection through their ability to remain in a dormant state were pushed to enter the cell cycle and then became a target to be killed by OBP-301.

Oncolytic Herpes Simplex Viruses

The oncolytic herpes simplex virus (OncoHSV) belongs to the neurotropic virus category, which is primarily effective against cancers of the nervous system (Li et al. 2020). For example, the FDA has authorized a phase III trial to treat melanoma using the oncolytic virus named T-VEC, which is administered either alone or in combination with other medical therapies (Bommareddy et al. 2017). Even if the OncoHSV is specific for neurological malignancies, their efficacy has also been tested in colon cancer with encouraging data. There was a selective killing of the cancer stemlike cells in vitro and in vivo (Bommareddy et al. 2017; Hill and Carlisle 2019). As for the cancer of the nervous system, the designed OncoHSV named YE-PC8, which regulates the cell cycle, was responsible for 80% of the tumor regression in glioma (de Sostoa et al. 2019; Kahramanian et al. 2019; Dmitrieva et al. 2011; Mckee et al. 2006; Guedan et al. 2010; Desjardins et al. 2018). Regarding metastatic gastric cancer resisting the standard chemotherapy treatments, the use of OncoHSV NV1020 was considered as a novel treatment option and led to an overall improved survival during a phase I clinical study due to reduced tumor growth (https://clinicaltrials.gov/ct2/show/NCT00149396). Also, another OncoHSV directed against gastrointestinal cancer showed promising results avoiding side effects (Yang et al. 2016). A recent study has demonstrated that another OncoHSV was potent in the killing of glioma CSCs (Kambara et al. 2005). This oncovirus, developed by the Kambara laboratory, allowed nestin expression in the embryonic neuroglial cell inhibiting tumor growth (Kambara et al. 2005).

Oncolytic Virus and Its Effects in Recent Clinical Trials

Recent preclinical studies have investigated OVs' role in solid tumors using an experimental pancreatic model (Eissa et al. 2018). Besides that, these models are complex and are only used for a specific virus; the oncolytic ONYX-015 adenovirus has already been tested in phase I and phase II clinical trials. ONYX-015 (dl1520) is an E1B-55 kDa gene-deleted virus replicating selectively in p53-mutated tumor cells (Mulvihill et al. 2001). During the phase I clinical trial, 23 patients with inoperable pancreatic cancer were enrolled to receive ONYX-015 at 4 weeks by a computed tomography injection. Most of the patients responded well and gave encouraging data, except for one patient who developed pancreatitis. No adverse effects were reported in the study; however, no objective response against the tumor was observed during the phase I study. In order to enhance the effectiveness of ONYX-015 therapy, phase II clinical trials were designed involving the use of a combination of ONYX015 with gemcitabine (Mulvihill et al. 2001). A total of 21 patients with advanced and metastatic pancreatic adenocarcinoma were enrolled in the phase II study (Mulvihill et al. 2001). Phase II studies reported two partial regressions and two minor responses without any severe side effects. Some more clinical trials are currently underway to assess VCN-01 and LOAd703, preparations either alone or in combination with nab-paclitaxel/gemcitabine in metastatic pancreatic cancer (NCT02705196, NCT02045589, and NCT02045602).

Oncolytic Adenoviruses and Pancreatic Clinical Trials

Preclinical studies investigating the role of OVs in solid tumors have been recently set. In particular, the pancreatic model has been used. Althought these models are complex and developed only for a specific virus, the oncolytic ONYX-015, specific for p53-mutated cells (Mulvihill et al. 2001). In a phase I dose-escalation clinical study, 23 patients have been enrolled with a diagnosis of inoperable pancreatic cancer. ONYX-015 was administered every 4 weeks by a computed tomography injection, and the results were generally satisfying except for one patient who developed an episode of pancreatitis. Although the results seem to be without adverse effects, no objective response against the tumor was observed in the phase I clinical study; however, most of the patients showed low level of cellular immunity (indicated by low number of CD4 cells). Moreover, no viral replication was observed in any of the patients receiving ONYX-015.

In the light of these data, a combination of ONYX015 and gemcitabine was used during a subsequent phase II clinical study involving 21 patients with advanced and metastatic pancreatic adenocarcinoma without liver metastasis (Hecht et al. 2003). The trial was designed to ascertain the safety, feasibility, and tolerability of the combinatorial therapy approach of chemovirotherapy. The authors reported partial regression of the injected tumors in two patients, while another two patients showed minor responses with no severe side effects. The remaining patients either had a

stable disease progression or had to leave the study due to treatment intolerance. Other two clinical trials are running at the moment (VCN-01 and LOAd703), either alone or in combination with nab-paclitaxel/gemcitabine in metastatic pancreatic cancer (NCT02705196, NCT02045589, and NCT02045602).

Oncolytic Herpes Simplex Viruses and Pancreatic Clinical Trials

Concerning therapies using herpes simplex viruses, seven viruses were selected for their assessment during phase II clinical trials for different solid tumors (Table 2). Only HF10 and T-VEC were studied during these trials to treat pancreatic cancer. During a single-arm, open-label phase I clinical study, eight patients were enrolled to receive HF10 intra-tumoral injection of HF10 during surgery. Each patient received injection every 2 weeks for up to 4 weeks. The results of the study showed that three patients achieved stable condition; one showed partial remission, while other showed a progression. Three patients showed an increase in the tumor-specific marker CA19.9. Histological studies showed that HF10 activated macrophages as well as natural killer (NK) cells (Hirooka et al. 2018; Nakao et al. 2011). However, the results failed to show any significant remission of the disease despite that molecular connections are being identified between CSCs and macrophages as part of the novel strategies to treat cancer (Aramini et al. 2021). Another clinical trial studying unresectable pancreatic cancer is in progress to test the safety and efficacy of HF10 in combination with gemcitabine, nab-paclitaxel, or S-1 (tegafur/gimeracil/oteracil, TS-1®) (NCT03252808) (https://clinicaltrials.gov/ct2/show/NCT03252808). Two more clinical trials were designed after encouraging data from phase I clinical trial

Table 2 MicroRNAs and oncolytic viruses for possible future therapy in solid tumors: preclinical results

Organ	miRNAs [Ref.]	Oncolytic virus [References]
Lung	miRNA-106b-5p Yu et al. (2017)	Ad5D24-CpG Garofalo et al. (2018)
	miRNA-7 Zhao et al. (2015)	VSV-IFNβ Patel et al. (2020)
	miRNA-99 Yu et al. (2015)	MYXV Kellish et al. (2019)
	miR-218-5p Zhu et al. (2016)	H101 Lei et al. (2015)
Breast	miR-142-3p Troschel et al. (2018)	MG1 Bourgeois-Daigneault et al. (2016)
	miRNA-150 Isobe et al. (2014)	G47Δ-mIL12 Ghouse et al. (2020)
	miR-203 Muhammad et al. (2016)	MRB Mullins-Densereau et al. (2019)
	miR-8084 Gao et al. (2018)	NDV Al-ZIyadi et al. (2020)
Brain	miR-21, miR-221 Wang et al. (2015)	DNX-2401, PVS-RIPO, Toca 511 Martikainen and Essand (2019)
		H-1PV Geletneky et al. (2017)
Thyroid	miR-21, miR183, miR-375 Chu and Lloyd (2016)	*dl*922-947 Heise et al. (2000)
		NV1020 Jiang et al. (2018)
		VB-111 Reddi et al. (2011)
Ovary	miR-628-5p (171)	CRAd-S-pk7 Mooney et al. (2018)

with T-VEC in 17 patients with pancreatic cancer. T-VEC doses were well tolerated, however, without an expected objective response (Conry et al. 2018).

Stem Cell-Derived Exosomes as Therapeutic Carriers

During the last decade, novel approaches in cancer cell targeting have been found and mainly involved microRNAs (miRNAs) and CSCs (Wang et al. 2011; Khan et al. 2010). Despite the successful outcome from several studies, a secure target regarding miRNAs or CSCs in solid tumors has not been identified yet. MiRNAs are small noncoding molecules able to regulate at least 60% of human genes, their expression, and hence their downstream signaling that affects diverse cellular functions ranging from metabolism to proliferation and differentiation. Hence, they are being used as part of the theranostic strategies (Haider and Aramini 2020). Like any other cell type, they are critical determinants of stem cell functions encompassing survival to proliferation of stem cells (Kim et al. 2010, Lai et al. 2011, 2012). Changes in miRNA profile also enhance solid tumors' induction and alter CSC stemness properties (Ruggieri et al. 2019; Aramini et al. 2020b). As previously described, CSCs appear to be involved in many processes regulating tumor and are often associated with poor clinical outcomes (Aramini et al. 2020b). One of the most exciting aspects recently studied miRNAs' role in CSC regulation and preservation through pathways as wingless (WNT)/β-catenin, Notch, JAK/STAT, PI3/AKT, etc. (Wang et al. 2011). These data are reckoned as a milestone for the identification of new target treatments against cancer. In particular, we have observed the association between miRNAs and their effects on CSCs' characteristics: self-renewal capacity, ability to differentiate, involvement in the metastatic process, etc. These data seem to be linked with several solid tumors. More recently, Khan et al. have reported an altered mRNA profile with a selective reduction in the number of miRNAs associated with CSCs, which may be a vital aspect in discovering new treatment targets (Khan et al. 2010).

Several miRNAs have emerged as potential oncogenic targets with a double role as tumor suppressors and tumor inductors. In breast cancer, miR-200 interacts with the tumorigenic process negatively, while members of miR-34 facilitate cell processes and apoptosis. In different solid cancers, downregulation of miR-34 and let-7 is responsible for oncogenesis, although their upregulation stimulates the suppression of cancer development (Khan et al. 2010; Ruggieri et al. 2019; Aramini et al. 2020b; Diana et al. 2019). A similar oncogenic activity has been confirmed for other miRNAs, i.e., miR-181 and miR-155, which seem to promote self-renewal, the formation of niche, and tumor development and progression.

In prostate cancer, downregulated expression of miRNAs, i.e., miR-34a, let-7b, miR-106a, and miR-141, has been observed in CSCs, whereas other miRNAs, i.e., miR-301 and miR-452, were highly expressed (Wang et al. 2011; Khan et al. 2010). Moreover, altered expression of miRNAs has been reported in breast cancer CSCs, with downregulation of miR-203 and miR-375 and concomitant upregulation of

miR-100, miR-221, miR-222, and miR-125b (Diana et al. 2019; Howard and Yang 2018; Ding et al. 2019). We summarize here the most active miRNAs involved in CSC regulation in different solid tumors (Table 2). These clinical trials focused on pancreatic cancers, and only HF10 and T-VEC were studied. During phase I clinical trials, eight patients were enrolled to receive an intra-tumoral injection of HF10 during surgery, with a stable condition in three patients, partial remission in one, and a progression in the others. Three patients showed an increase in tumoral marker CA19.9. Histologically, HF10 activated macrophages besides the activation of NK cells (Lim et al. 2019). However, the results do not show an improved remission of the disease. Another recent trial in unresectable pancreatic cancer is in progress to test the safety and efficacy of HF10 plus gemcitabine and nab-paclitaxel and S-1 (tegafur/ gimeracil/oteracil, TS-1®) (NCT03252808). The other two trials have been set without particular results, as expected, as phase I trial with T-VEC in 17 patients with pancreatic cancer. T-VEC doses were tolerated well; however, no objective response has been shown (Nakao et al. 2011).

Conclusions and Future Directions

Besides our knowledge about the significance of miRNAs, CSCs, and OVs for developing future targeted anticancer therapies, the prospect for these treatments' efficacy remains less well defined. This is primarily attributed to the nonavailability of optimal and standardized isolation and purification protocols and the shortage of specific CSCs' specific markers. Secondly, the unreliability of virotherapy continues to be problematic. Thirdly, there are many instances wherein both upregulation and downregulation of the same miRNAs occur in different types of tumors, signifying the importance of altered miRNA profile for specific miRNAs. The scientific community has a big responsibility in optimizing these new approaches for future generations of patients who will develop solid tumors. Of course, new innovative thinking is desirable; nevertheless, improved life quality in terms of duration and well-being must be considered in the clinical process. Several clinical trials are underway regarding the possibility of finding a practical approach in treating advanced cancers. However, the future of the oncological field is mainly focused on the prevention of cancer recurrence at early stages and prediction when and if cancer will return. In conclusion, the actual results regarding the role of CSCs in oncogenesis are currently less than satisfactory and therefore necessitate further in-depth investigations in the future.

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Bioengineering Technique Progress of Direct Cardiac Reprogramming

45

A New Perspective from Microbubbles and UTMD

Dingqian Liu, Khawaja Husnain Haider, and Changfa Guo

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D. Liu · C. Guo (✉)
Department of Cardiac Surgery, Zhongshan Hospital, Fudan University, Shanghai, People's Republic of China
e-mail: dqliu10@fudan.edu.cn; guo.changfa@zs-hospital.sh.cn

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

K. H. Haider (ed.), *Handbook of Stem Cell Therapy*,
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Abstract

The combination of heart-specific transcription factors GATA4, MEF2C, and TBX5 (GMT) has been proven to have the ability to directly reprogram cardiac fibroblasts; this approach is considered a promising regenerative technique. Meanwhile, research on microbubbles as biological vectors has made great progress in recent years. This study describes the loading of GMT lentiviral vectors on cationic microbubbles and the release of these direct-reprogramming vectors into an infarcted myocardium by ultrasound targeted microbubble destruction (UTMD) to repair the cardiac tissue. Lentivirus which encode GATA4, MEF2C, and TBX5 transcription factors were generated via a lentiviral production system and were confirmed to have a direct reprogramming ability in vitro. Combined with the cationic microbubbles, UTMD-mediated gene delivery was evaluated, and the gene transfection efficiency was optimized in an in vitro experiment on rat cardiac fibroblasts. With UTMD-mediated direct reprogramming, the viral vector particles were directly deposited in cardiac tissue and repaired the infarcted myocardium. An immunofluorescence assay and histological examination confirmed newborn cardiomyocytes and neo-angiogenesis after a 4-week follow-up of the treated rats. All treated groups showed ventricular-function improvement according to cardiac magnetic resonance imaging and echocardiography. This chapter reports a novel strategy for the delivery of direct-reprogramming lentiviral vectors to a target acute myocardial infarction zone by using UTMD as a tissue repair therapy.

Keywords

Bioengineering · Cardiac · Cationic · Direct reprogramming · iPSCs · Ischemic heart disease · Microbubbles · Myocardium · Stem cells · UTMD

Abbreviations

CFs	Cardiac fibroblasts
cTnI	Cardiac troponin-I
cTnT	Cardiac troponin-I
GMT	Gata4, MEF2c, and TBX5
H&E	Hematoxylin and Eosin
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
iCMs	Induced cardiomyocyte-like cells
IHD	Ischemic heart disease

iPSCs	Induced pluripotent stem cells
LAD	Left anterior descending
LV	Left ventricle
LVEF	LV-ejection fraction
MCAs	Microbubble ultrasound contrast agents
MI	Myocardial infarction
UTMD	Ultrasound-targeted microbubble destruction

Introduction

Ischemic heart disease (IHD) is the leading cause of heart failure (Moran et al. 2014). According to the recently published report by the American Heart Association, cardiovascular diseases incur one of the highest health and economic burdens globally (Virani et al. 2021). Although the contemporary surgical and pharmacological options provide symptomatic relief to the patients in most cases (Santucci et al. 2020), these treatment options fail to recover the ischemic damage incurred at the structural and cellular levels in terms of massive cardiomyocyte loss. Heart transplantation is widely used as a gold-standard treatment option; organ transplantation still has disadvantages, including an insufficient number of donor organs and the need for long-term immunosuppressant drug therapy (von Dossow et al. 2017). Given that adult cardiomyocytes have only limited regenerative capacity (Bergmann et al. 2009; Mollova et al. 2013), the intrinsic repair mechanism by the resident cardiac stem cells to recover the massive loss of functioning myocytes is only restricted and less than optimal. Although protocols have been reported to reenter the surviving cardiomyocytes in the peri-infarct region into the cell cycle or stimulate the resident stem cells to participate more efficiently in the repair process, the success has been limited (He and Zhou 2017). Attempts have been made to directly reprogram cardiac fibroblasts by myocardial delivery of GATA4, Mef2, and Tbx5 to adopt cardiomyocyte phenotype in experimental animal studies (Qian et al. 2012).

Alternatively, stem cell-based therapy has a promising future in cardiac regenerative medicine. Since the publication of the first report of cell-based therapy in a 57-year-old patient undergoing coronary artery bypass grafting (Menasché et al. 2001), the novel stem cell-based intervention has progressed from bench to bedside, advancing to multicenter randomized Phase II and Phase III clinical trials, primarily focusing on the use of bone marrow-derived stem cells (Heldman et al. 2014; Mathiasen et al. 2015; Hare et al. 2017). Despite the well-established safety aspects of cell-based therapy for myocardial repair in preclinical and clinical studies, the efficacy in most cases, especially in clinical studies, has been modest. These modest clinical data have been ascribed mainly to the cell-type selection and the quality of the cells used for transplantation (Shahid et al. 2016; Haider 2018). In this regard, pluripotent stem cells, mainly induced pluripotent stem cells (iPSCs), have given new impetus to cell-based therapy.

Pluripotent Stem Cells in Myocardial Regeneration and Repair

From among the pluripotent stem cells, undifferentiated embryonic stem cells (ESCs) and their derivative cardiomyocytes have been extensively studied in experimental animal models for myocardial repair and regeneration. The transplanted cells differentiate into morphofunctionally cardiomyocytes, endothelial cells, and vascular smooth muscle cells as part of their mechanism to regenerate infarcted myocardium and preserve global cardiac function (Min et al. 2002; Nelson et al. 2006; Caspi et al. 2007; Rufaihah et al. 2007, 2010; van Laake et al. 2008). However, ESCs have not progressed beyond preclinical experimental studies due to moral and ethical issues associated with their availability and use, and tumorigenic potential. Pre-differentiation strategy of ESCs to form cardiomyocytes rather than using undifferentiated ESCs has been pursued to address the tumorigenicity concerns. However, the contaminant population of undifferentiated or incompletely differentiated cells still remains a concern for in vivo use. Hence, various genetic and nongenetic methods of selection and enrichment of pluripotent stem cell-derived cardiomyocytes have been reported (Lin et al. 2010; Ban et al. 2013, 2015). However, all this is still in experimental phase.

In 2006, Takahashi and Yamanaka were the first to successfully generate iPSCs from adult somatic cells by integrating-virus-based delivery of a classic combination of four transcription factors, i.e., Oct3/4, Sox2, cMyc, and Klf4 (Takahashi and Yamanaka 2006). The reprogrammed cells were pluripotent and were considered as surrogate ESCs due to similarity in biological and stemness characteristics, however, without ethical and moral strings associated with the production and application of ESCs (Ibrahim et al. 2016). Although this groundbreaking discovery gave new impetus to stem cell-based therapy, tumorigenicity during preclinical assessment raised serious concerns about this progress to the clinical use in the patients (Ahmed et al. 2011; Buccini et al. 2012).

Various strategies have been adopted to generate iPSCs to fully exploit their theranostic applications from drug development to disease modelling in vitro. They are also suitable for patient use in the clinic. These strategies include the use of a smaller number of transcription factors, mainly the exclusion of cMyc (Nakagawa et al. 2008, 2010; Giorgetti et al. 2009), the use of non-integrative viral vectors (Chou et al. 2011; Macarthur et al. 2012), and nonviral vectors for transcription factors' delivery (Huangfu et al. 2008; Qu et al. 2012). Somatic cell reprogramming protocols have also used a combination of small molecules and pluripotency-related transcription factors to overcome the low efficiency of classical transcription-based protocols. For example, DNA methyltransferase and histone deacetylase (HDAC) inhibitors, i.e., valproic acid, the treatment improved the efficiency of reprogramming by 100-fold (Huangfu et al. 2008; Pasha et al. 2011). Similarly, Woltgen et al. successfully used TGF-β inhibitors E-616452 (25 mM), E-616451 (3 mM), or EI-275 (3 mM) for induction of pluripotency in mouse embryonic fibroblasts (MEF) (Woltjen and Stanford 2009).

Although the overexpression of transcription factors for reprogramming is the most prevalent method for cellular reprogramming, search for novel meth ods with higher efficiency and safety index continues. Given their essential participation in major cellular signaling pathways involved in various cellular functions, an exciting strategy for somatic cell reprogramming is based on miRNA or a set of miRs manipulation to achieve cellular reprogramming. For example, Anokye-Danso and colleagues reported that expression of miR-302/miR-367 cluster, ESCs-specific miRs highly expressed during the embryonic development, successfully and efficiently reprogrammed both human and mouse somatic cells such that reprogramming efficiency were two orders of the magnitude more efficient than the classical transcription based-method (Anokye-Danso et al. 2011). Also, there was no difference in the quality and characteristics of the iPSCs generated from the two methods. More importantly, the strategy alleviated the need to deliver transcription factors to the cells. An important step forward in cellular reprogramming is the application of CRISPR/Cas9 technology, a gene editing technique which works as molecular scissors (Shakirova et al. 2020).

The Strategy of Direct Reprogramming to Generate Cardiomyocytes

Direct reprogramming is an emerging new approach which is being used to derive cardiomyocytes from differentiated somatic cells without passing through pluripotency status (Kelaini et al. 2014). The strategy generally involves developing partially induced multipotent progenitors which are then directed to differentiate into cardiomyocytes (Kelaini et al. 2014). For example, Ieda et al. successfully used direct reprogramming strategy to convert mouse cardiac fibroblasts (CFs) into induced cardiomyocyte-like cells (iCMs) using three heart-specific transcription factors: GATA4, MEF2C, and TBX5 (GMT) (Ieda et al. 2010; Kurotsu et al. 2017). This bioengineering technique is an exciting approach free from the limitations of the existing technologies, such as exogenous stem cell injection or iPSCs. Similar approach has also been used by other research groups using different sets of heart-specific transcription factors (Song et al. 2012; Protze et al. 2012; Mathison et al. 2012).

Microbubble ultrasound contrast agents (MCAs) have become an effective tool for gene delivery (Negishi et al. 2016). MCAs' biocompatible shells make them stable carriers of transfection vectors for gene delivery (Sun et al. 2013; Panje et al. 2012; Geis et al. 2012). With a compressible gas core, MCAs have a unique feature that allows them to be flexible according to different phases of ultrasound waves (Shapiro et al. 2016). In addition, the cell membranes and vascular endothelial integrity can be trespassed by the ultrasound shock waves. Hence, MCAs can penetrate vessels in an ultrasound field and serve as therapeutic modality for the circulatory system (Castle and Feinstein 2016; Chen et al. 2016). Recent research

yielded a reliable method for fabricating controlled-release vectors loaded with MCAs; this method is ultrasound-targeted microbubble destruction (UTMD) (Zhou et al. 2017). High-amplitude oscillations leading to microbubble destruction can be induced by high-mechanical-index ultrasound at its resonance frequency (Liao et al. 2014). This sonoporation phenomenon improves localized tissue deposition of bioactive substances. Additionally, the features mentioned above, UTMD generates high-velocity fluid microjets that produce transient nanopores in cell membranes and enable higher capillary permeability (Haber et al. 2017). Considering UTMD as a feasible targeted delivery method, some authors have successfully loaded vectors, including viral vectors and plasmid DNA to treat a wide range of diseases in different animal models (Zhang et al. 2018; Lin et al. 2018).

The virus-mediated expression cloning methods include retroviral, Sendai virus, and lentiviral vector-mediated gene delivery systems. The characteristics of these viral systems, i.e., high transduction rates, make them popular in stem cell research (Sharon and Kamen 2018; Gandara et al. 2018; White et al. 2017; Miyamoto et al. 2018; Isomi et al. 2021). Nowadays, the HIV-1–derived third-generation lentiviral vector is the most commonly used manufacturing system, which originates from the laboratories of Didier Trono and David Baltimore (Kotterman et al. 2015). After deletion of two-thirds of the HIV-1 viral genome and preservation of the elements critical for gene transfer, this viral packaging system is free from replication competence risk. As a result, the biggest advantage of this system is replication deficiency, making lentiviral vectors safe for further applications in mammalian tissues.

Many clinical trials using lentiviruses for gene therapy have been conducted in recent years (Houghton et al. 2015; Liu et al. 2014; Aiuti et al. 2013). The results proved that lentiviral vectors could facilitate the delivery of genetic material and its stable long-term expression in a target tissue without severe inflammatory response. In polar media, viral vectors also have a pH-dependent surface charge. As a result, the electrostatic charge of virus particles, also known as the isoelectric point, has an important property in an electric field (Michen and Graule 2010). This property determines the viral electrostatic adsorption capacity, which is critical for further virus sorption processes. Based on this property of viral vectors, injection of cationic microbubbles (CMBs) loaded with viral vectors affords an improved gene transfection effect in vivo (Li et al. 2009; Xie et al. 2010). Some studies have also reported successful delivery of MCA–virus hybrids to the cardiac tissue, indicating the feasibility of organ-specific targeting of viral vectors (Michen and Graule 2010).

Inspired by direct-reprogramming strategies and the research advances in MCAs, we have attempted to synthesize CMBs so that lentiviral vectors encoding GMT for direct reprogramming could be loaded on the surface of microbubbles (Fig. 1). Instead of direct intramyocardial injection, the microbubble–lentivirus hybrids were injected through the jugular vein, and direct-reprogramming relevant vectors were then released into the targeted myocardium by UTMD. This chapter is based on our novel data and discusses in-depth the superiority of this novel approach in regenerative medicine.

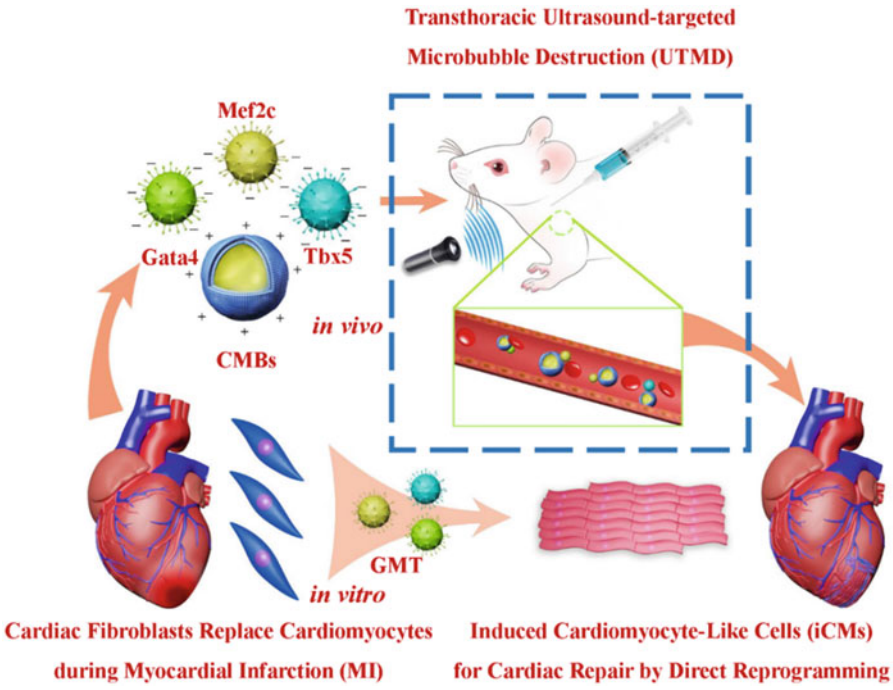

Fig. 1 A schematic of ultrasound-targeted CMB destruction–mediated localized delivery of the direct-reprogramming GMT-encoding lentivirus for cardiac repair in ischemic heart disease. The lentivirus encoding heart-specific transcription factors, GATA4, MEF2C, and TBX5 (GMT) have the capacity to directly reprogram CFs into iCMs in vitro. The synthesized CMBs are loaded with an anionic GMT lentiviral vector so that the microbubble/lentivirus hybrids could be then injected via the jugular vein, and direct-reprogramming vectors are released into the targeted myocardium by the UTMD technique for cardiac repair in vivo

Observations and Data Analysis

Synthesis and Characterization of CMBs and Lentivirus-Binding Capacity

During the study, CMBs were developed using DPPC, DPPE, and DOPE in chloroform, followed by rotary evaporation to form a lipid film (Fig. 2a–c). After sufficient rehydration with PBS and emulsification with glycerine, the resulting emulsion was sonicated with a gas (SF_6). Thus, the CMBs with a sulfur hexafluoride gas core and a lipid shell were fabricated. The concentration of CMBs was approximately 4.0×10^9/ml. The average microbubble diameter was 966.6 nm (Fig. 2d), and the zeta potential of the CMBs was 28.3 ± 1.4 mV (Fig. 2e).

The initial mean microbubble count in this experiment was $6.1 \pm 0.2 \times 10^7$. Given the surface of CMBs was cationic and the lentivirus capsids were anionic, the

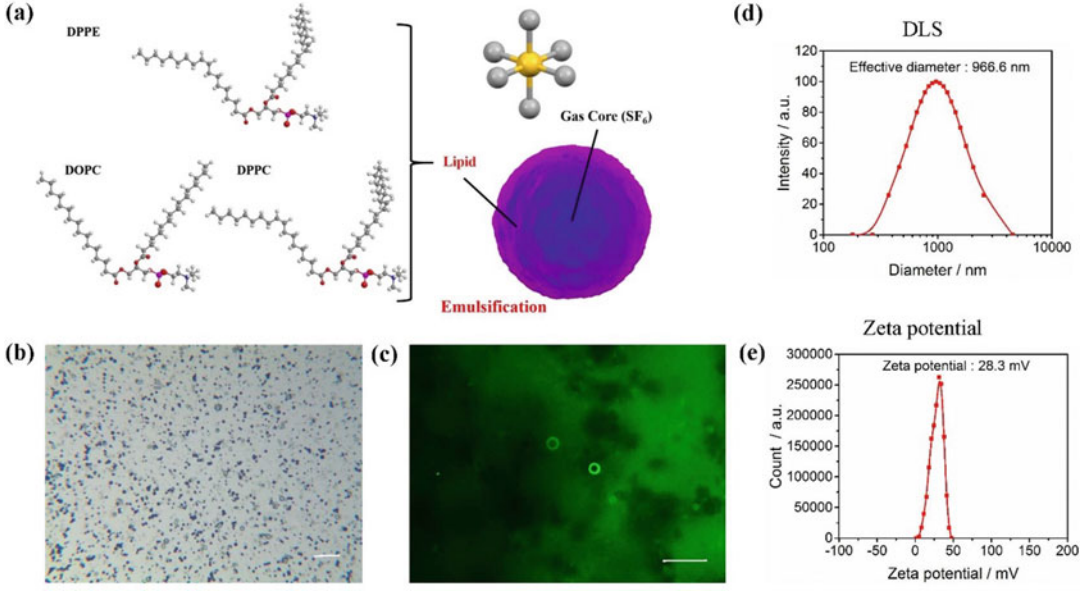

Fig. 2 Design and characterization of CMBs. (**a**) A schematic of CMB synthesis. The CMBs consist of DPPC, DPPE, and DOPE in chloroform, and the CMBs with an SF_6 gas core and a lipid shell are fabricated. (**b**) The shape and concentration of CMBs were evaluated microscopically, and CMBs appeared as gas-filled spheres under a microscope. (**c**) Microbubbles loaded with the control GFP-expressing lentivirus were labelled with the FITC-conjugated anti-p24 antibody, revealing a strong fluorescence signal on the microbubble surface, which indicated that the GFP-expressing lentivirus was bound to the surface of microbubbles. (**d**) Log-normal size distribution and (**e**) zeta potential of the CMBs as measured by a Zetasizer NANO ZS system

loaded microbubbles could be distinguished from the unloaded ones by fluorescence to prove viral-vector binding. As microbubbles have low stability upon exposure to repetitive flotation or gentle shaking, some of the original microbubbles could be retrieved after all the washing steps. After incubation with the FITC-conjugated anti-p24 antibody, the loaded microbubbles showed a strong fluorescence signal on their surface, thus proving that anionic capsids of the GFP-expressing lentivirus were efficiently bound to the microbubbles' surface (Fig. 2c).

Reprogramming of Rat CFs into iCMs by the GMT Lentivirus Vectors In Vitro

Amplified and cloned sequences of *Gata4*, *Mef2c*, and *Tbx5* in the plasmids for lentiviral packaging were confirmed by restriction enzyme identification and gene sequencing (Fig. 3a). After packaging, the titer of GATA4 and TBX5 viral vectors were 3.05×10^8 and 3.02×10^8 TU/ml, respectively, according to the results of 293 T cells infection. The titer of the MEF2C vector was 3.25×10^8 TU/m as confirmed by an RT-PCR assay (Table S1). After infection of 293 T cells, the infected cells showed the corresponding fluorescence signal and puromycin resistance, which confirmed the successful construction of reprogramming vectors (Fig. S1).

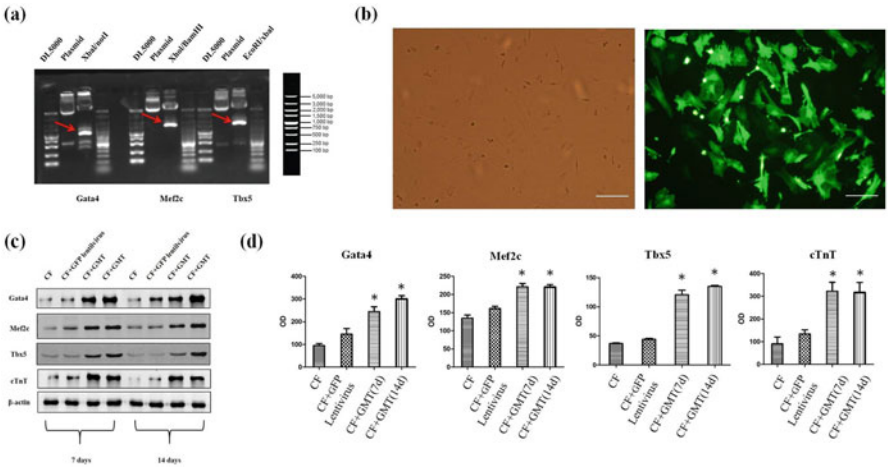

Fig. 3 Optimization of in vitro reprogramming. (**a**) Restriction enzyme identification and gene sequencing confirmed amplified and cloned sequences of *Gata4*, *Mef2c*, and *Tbx5* in the plasmids for lentivirus packaging. (**b**) At the fixed parameters (1.5 MHz, duty cycle 20%, duration 30 s), the EGFP-positive cells of the 1.5 W/cm^2 ultrasound group were detected by fluorescence microscopy. (**c**) A representative Western blot showing overexpression of all three transcription factors GATA4, MEF2C, and TBX5, and cardiomyocyte-specific marker cTnT at 7 and 14 days after lentivirus infection (*$P < 0.05$ vs. control)

Table S1 Real-time PCR sequences for detecting Mef2c lentiviral vector titer

WPRE sequence	
Forward primer (1277F)	CCGTTTCAGGCAACGTG
Reverse primer (1361R)	AGCTGACAGGTGGTGGCAAT
Probe(1314P)	FAM-TGCTGACGCAACCCCCACTGGT-TAMRA
β-actin sequence	
Forward primer	GCGAGAAGATGACCCAGCTC
Reverse primer	CCAGTGGTACGGCCAGAGG
Probe	FAM-CCAGCCATGTACGTTGCTATCCAGGC-TAMRA

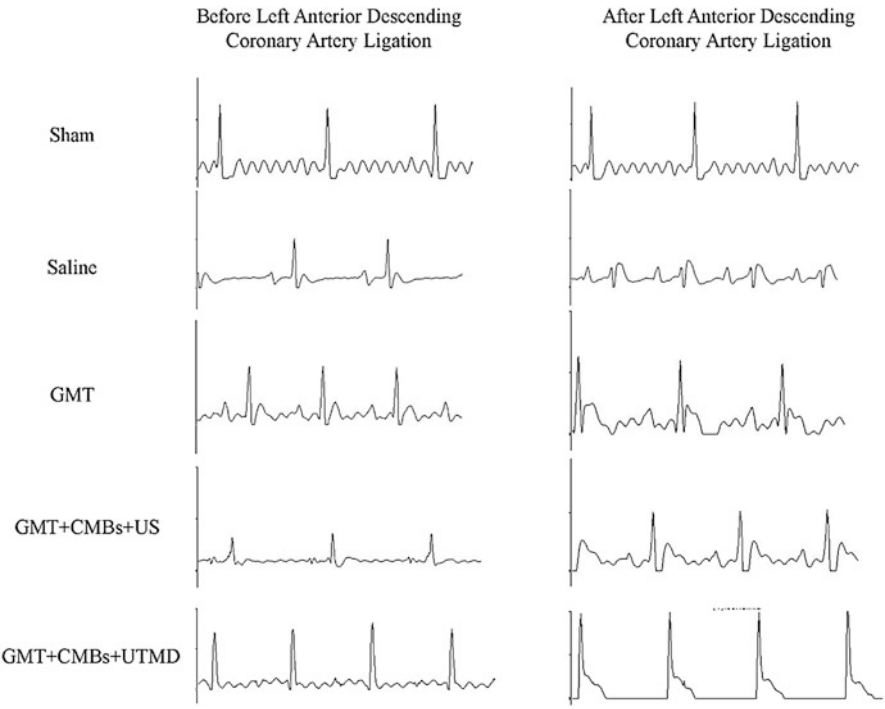

Figure S1 Electrocardiogram (ECG) of the left anterior descending coronary artery (LAD) rat model. Line 1: ECG of shame group ($n = 8$) before and after LAD ligation; Line 2: ECG of saline group ($n = 8$) before and after LAD ligation; Line 3: ECG of GMT group ($n = 8$) before and after LAD ligation; Line 4: ECG of GMT+CMBs+US group ($n = 8$) before and after LAD ligation; Line 5: ECG of GMT+CMBs+UTMD group ($n = 8$) before and after LAD ligation

After in vitro cell transfection with the GMT viral vectors, Western blotting revealed overexpression of all three reprogramming transcription factors after 7 days. The expression level remained stable until day 14 of observation (Fig. 3c, d). In addition to the increased expression of the three reprogramming transcription factors, overexpression of the cardiomyocyte-specific marker cTnT was confirmed by Western

blotting after the transfection. In contrast to the infected CFs, those infected with the control vectors did not show the same expression levels of transcription factors or cTnT. Therefore, the fabricated GMT viral vectors were capable of in vitro reprogramming of CFs, which expressed the typical marker of cardiomyocytes.

UTMD-Mediated Localized Delivery of Direct-Reprogramming GMT Lentivirus–CMB Hybrids In Vivo

Histological Assessment of Post-Infarct Ventricles After UTMD Therapy

Neomyogenesis around the infarct area after GMT and UTMD therapy via potential cardiomyocyte proliferation was demonstrated by immunofluorescence staining specific for α-actin and histone H3 phosphorylation (PHH3) expression, a marker of cell mitosis. Twenty-eight days after the surgical procedure, an increase in the PHH3-specific immunofluorescence signals were detected in the peri-infarct area in groups GMT, GMT + US, and GMT + UTMD treatment groups (Fig. 4, panels-2 & 4). Superimposition of immunofluorescent images of α-actin (Fig. 4, panel-1) and DAPI stained images (Fig. 4, panel-3), showed that treatment with GMT, GMT + US, and GMT + UTMD induced cardiomyocyte proliferation after myocardial infarction (MI). According to statistical analysis (Fig. S2), there were significantly more PHH3-positive and α-actin–negative cells in the peri-infarct zone in all the treated groups; these data fit the characteristics of reprogrammed fibroblasts under the conditions of MI.

The TdT-mediated dUTP nick-end labelling (TUNEL) analysis of the peri-infarct myocardium in groups GMT, GMT + US, and GMT + UTMD manifested a lower percentage of apoptotic cardiomyocytes than that in the saline treated group of animals ($P < 0.05$). Groups GMT, GMT + US, and GMT + UTMD showed no statistically significant differences among themselves (Fig. 5a, panel-3, & Fig. 5d). It was observed that the damaged myocardium in the saline group was replaced by a fibrous scar (Fig. 5a, panel-2), and ventricular-wall thickening could be observed at 28 days after infarction. Masson trichrome staining and HE staining were also used (Fig. 5a, panels-2 & 3) to measure the scar size and quantitatively analyze interstitial fibrosis (Fig. 5b, c).

Compared with the saline treated group of animals, myocardial fibrosis leading to scar formation and left ventricular remodeling were significantly attenuated in all the treatment groups. The total area of fibrosis and the number of scar-producing myofibroblasts were significantly reduced observed after the administration of the GMT vectors at 28 days of observation. In addition, angiogenesis, which is important for restoration of regional blood supply (including capillaries and arterioles), was significantly improved in the peri-infarct area. In the groups GMT, GMT + US, and GMT + UTMD, there was significant increase in capillary density after immunostaining for cluster of differentiation 31 expression (CD31; Fig. 5a, panel-5 and Fig. 5e), and arteriole counts [according to combined staining for alpha-smooth muscle actin (α-SMA) in the peri-infarct zone] than the saline treatment group (Fig. 5a, panel-4, and Fig. 5f). Although without statistical significance, immunohistochemical

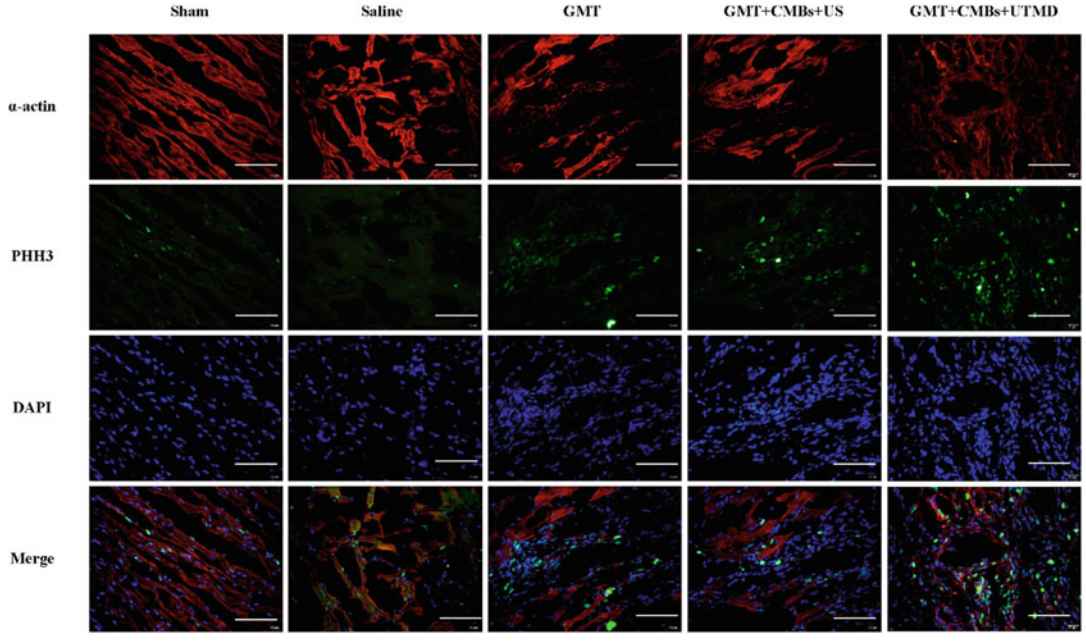

Fig. 4 Neomyocytes around the infarct area after in vivo reprogramming. Panel 1: Immunofluorescence of α-actin (red fluorescence). Panel 2: Immunofluorescence of PHH3 (green fluorescence). Panel 3: DAPI staining showing cardiomyocytes nuclei (blue fluorescence). Panel 4: Merged images showing that newborn cardiomyocytes can be detected via PHH3 expression by fluorescence microscopy 28 days after the operation (scale bar ¼ 50 mm)

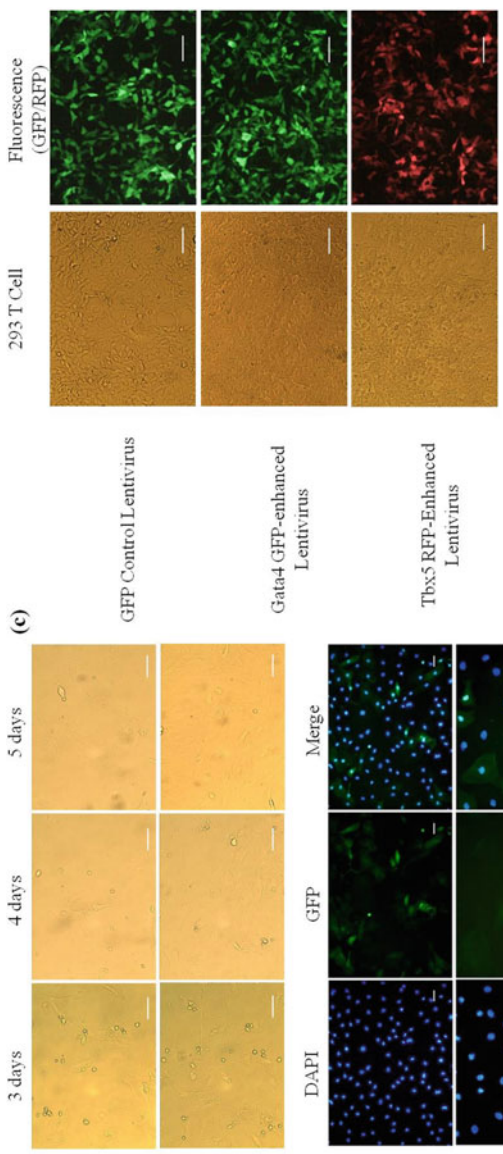

Figure S2 Screening experiments results of GMT lentivirus (**a**) The packaging plasmids of Mef2c consist of puromycin gene which provides transfected cells puromycin resistance. Line 1: Puromycin resistance experiment for fibroblasts. Line 2: Puromycin resistance experiment for fibroblasts transfected with GMT lentiviral vectors. (**b**) Cardiac fibroblasts infected by 10^8 TU/ml GFP control lentivirus. (**c**) Gata4 and Tbx5 lentivirus titers counting by infecting 293T human embryonic kidney cell. Line 1: 293T cells infected by GFP control lentivirus; Line 2: 293T cells infected by GFP-enhanced Gata4 lentivirus; Line 3: 293T cells infected by RFP-enhanced Tbx5 lentivirus

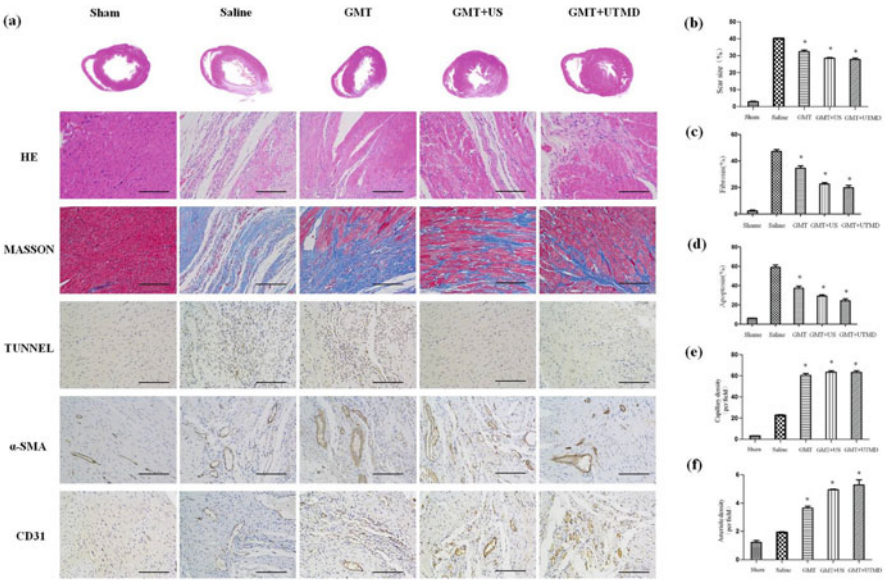

Fig. 5 Histological examination of ultrasound-mediated in situ direct reprogramming in the rat heart model. (**a**) Histological analyses. Panel 1: HE staining images depicting the basic tissue structure of the histological tissue sections 28 days post-surgery; Panel 2: HE staining illustrating the basic tissue structure in histological tissue sections 28 days post-surgery (scale bar is ¼ 50 mm); panel 3: Masson trichrome staining showing a scar (blue) and viable (red) tissue sections 4 weeks post-surgery (scale bar is ¼ 50 mm); panel 4: TUNEL staining showing apoptotic cardiomyocytes (brown) 1 day post-surgery (scale bar ¼ 50 mm); panel 5: immunohistochemical images of arterioles stained for α-SMA (brown; scale bar ¼ 50 mm); panel 6: immunohistochemical images showing capillaries stained for CD31 (brown; scale bar ¼ 50 mm). (**b**) Quantitative analyses indicating that the scar size in groups GMT, GMT + US, and GMT + UTMD at 4 weeks post-surgery was smaller than that in any other group. (**c**) Quantitative analyses indicating that the fibrosis percentage in animal groups GMT, GMT + US, and GMT + UTMD 4 weeks post-surgery was less than the saline treated animal group. (**d**) Quantitative analysis suggesting that the apoptotic-cardiomyocyte percentages in the peri-infarct myocardium of groups GMT, GMT + US, and GMT + UTMD 28 days post-surgery were lower as compared to the saline treated group of animals. (**e**) Quantitative analysis showing that capillary density at 4 weeks post-surgery in the peri-infarct myocardium of groups GMT, GMT + US, and GMT + UTMD was higher than the saline treated animal group. (f) Quantitative analysis indicating that arteriole counts in the peri-infarct myocardium of groups GMT, GMT + US, and GMT + UTMD at 4 weeks post-surgery were higher than the saline treated animal group (*$P < 0.05$ vs. saline group)

staining of CD31 and α-SMA indicated an increase in angiogenesis in the peri-infarct area at 4 weeks post-surgery in UTMD and GMT lentiviral-vector–treated groups.

Histological examination of related organs including lungs, the liver, kidneys, and spleen in all the groups was also performed 28 days after the intervention. HE staining showed delicate alveolar walls of the lungs without any signs of pathological changes such as edema or hemorrhage (Fig. 6, panel-1). The liver in all the groups examined appeared normal, divided into lobules with the central vein and peripheral triads (Fig. 6, panel-2). There was no enlargement of the white pulp or a

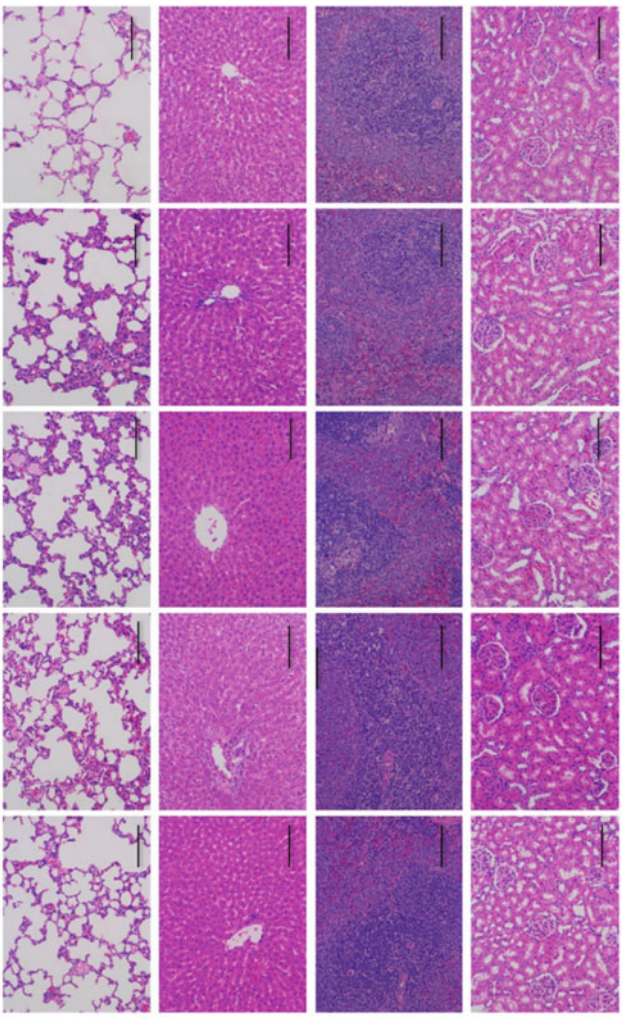

Fig. 6 Histological examination of various organs in all the groups at 28 days after respective intervention. Panel 1: HE staining images revealing the basic lung tissue structure of mass sections; panel 2: HE staining images showing the basic liver tissue structure of mass sections; panel 3: HE staining images revealing the basic spleen tissue structure of mass sections; panel 4: HE staining images depicting the basic kidney tissue structure of mass sections (all images scale bar ¼ 1 cm)

reduction in the red pulp composed of many splenic sinusoids in the spleen (Fig. 6, panel 3). The structures of glomeruli were also normal in all the kidneys (Fig. 6, panel-4). Immunohistochemical staining of GATA4, MEF2C, and TBX5 was also performed in the heart, liver, spleen, lung, and kidney tissues 28 days after the surgical procedure (Fig. S3), which revealed off-target expression in the liver and kidneys (Fig. S4). This was attributed to the high tropism of the lentivirus towards the liver and kidney cells.

Cardiac Function Improvement After UTMD-Mediated Direct Reprogramming Therapy

To assess the therapeutic effectiveness of GMT, GMT + US, and GMT + UTMD on global LV remodeling and function, serial echocardiographic analyses and cardiac MRI scans were performed on days at 2 and 28 after surgery in all the groups (Fig. 7a). MI via ligation of the left anterior descending coronary artery (LAD) induced a gradual reduction in LV-function, as evidenced by a declining LV-ejection fraction (LVEF), which is the critical event in the progression of adverse post-infarct remodeling. Two days post infarction, the results revealed similar baseline heart function among all the groups with induced infarction ($p > 0.05$). Compared with baseline (LVEF 46.3% ± 0.7%), the saline group showed significantly lower LVEF % (47.46% ± 2.9%) post infarction. On the other hand, treatment groups, including GMT, GMT + US, and GMT + UTMD had higher LVEF (54.01% ± 3%, 59.6% ± 1.9%, and 56.9% ± 1.98%, respectively; $p < 0.01$ vs. control group). However, all the treated groups showed no statistically significant difference in LVEF improvement among themselves (Fig. 7b–d). Therefore, cardiac function analysis from the baseline values showed that the LVEF after MI declined over time. As illustrated in the figure, the mean change in the LVEF in groups MI-CMB/GMT + UTMD, MI-CMB/GMT + US, and MI-CMB/GMT was more significant ($p < 0.05$) compared with the sham or saline group from the time point of lentivirus administration to the follow-up until 28 days of observation (Fig. 7b–d).

Significance of the Combinatorial Approach of UTMD with CMB-Lentiviral Hybrid

This data signifies the combinatorial approach of using UTMD with CMB–lentiviral hybrids to transfer direct-reprogramming GMT factors into rat CFs in the ischemic myocardium (Fig. 1). These data shows the high efficiency of ultrasound-targeted CMB destruction–mediated localized delivery of the direct-reprogramming GMT-encoding lentivirus for cardiac repair in ischemic heart disease. The lentivirus encoding heart-specific transcription factors, GATA4, MEF2C, and TBX5 (GMT) directly reprogrammed the non-cardiomyocytes population of cardiac cells, i.e., CFs, into iCMs in vitro successfully. The most significant findings of the presented data include successful neomyogenesis in and around the infarcted myocardium, increased blood vessel density, attenuated fibrogenesis and infarct size, and preservation of LV contractile function.

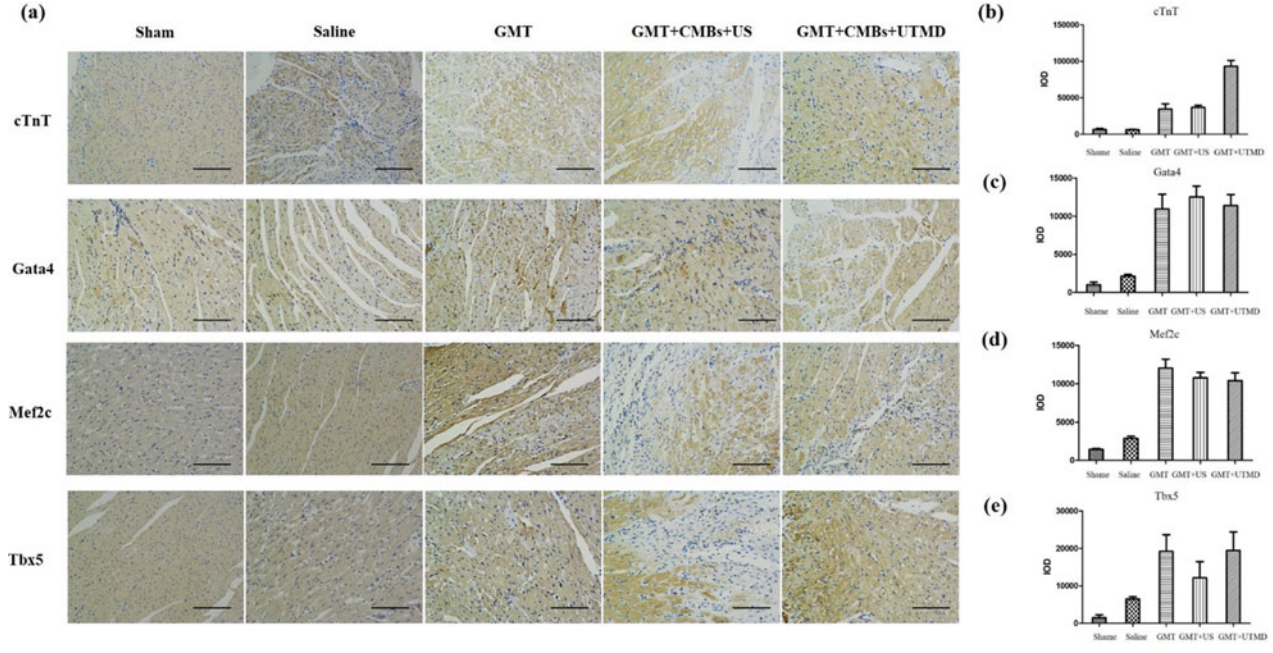

Figure S3 Histologic examination of ultrasound-mediated in situ direct reprogramming in rat model. (**a**) Histological analyses. Line 1: Immunohistochemistry images showing cTnT (brown), Scale bar ¼ 50 mm; Line 2: Immunohistochemistry images showing Gata4 (brown), Scale bar ¼ 50 mm; Line 3: Immunohistochemistry images showing Mef2c, Scale bar ¼ 50 mm; Line 4: Immunohistochemistry images showing Tbx5 (brown), Scale bar ¼ 50 mm. (**b**) Quantitative counting showing that cTnT positive counting 4 weeks post-surgery in peri-infarct myocardium of GMT, GMT+US, and GMT+UTMD groups are higher than saline group. (**c**) Quantitative counting showing that Gata4 positive counting 4 weeks post-surgery in peri-infarct myocardium of GMT, GMT+US, and GMT+UTMD groups are higher than saline group. (**d**) Quantitative counting showing that Mef2c positive counting 4 weeks post-surgery in peri-infarct myocardium of GMT, GMT+US, and GMT+UTMD groups are higher than saline group. (**e**) Quantitative counting showing that Tbx5 positive counting 4 weeks post-surgery in peri-infarct myocardium of GMT, GMT+US, and GMT+UTMD groups are higher than saline group

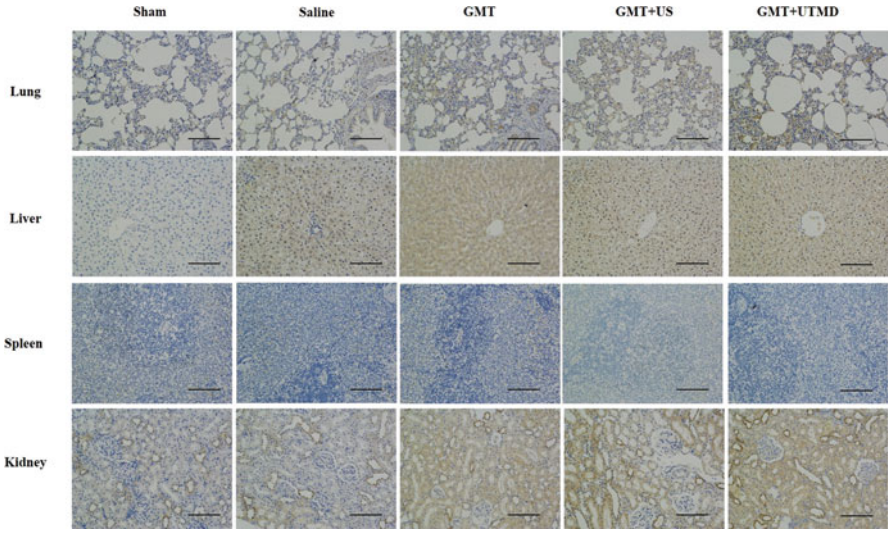

Figure S4 Immunohistochemistry stained by Gata4 in lung, liver, spleen, and kidney. Line 1: Immunohistochemistry images stained by Gata4 (brown) in lung tissue, Scale bar ¼ 50 mm. Line 2: Immunohistochemistry images stained by Gata4 (brown) in liver tissue, Scale bar ¼ 50 mm. Line 3: Immunohistochemistry images stained by Gata4 (brown) in spleen tissue, Scale bar ¼ 50 mm. Line 4: Immunohistochemistry images stained by Gata4 (brown) in kidney tissue, Scale bar ¼ 50 mm

CMs play a pivotal role in the structural repair maintenance of myocardial structural integrity of the infarcted myocardium by transforming into myofibroblasts. However, their excessive activation has been reported to support HF progression via fibrotic activity that contributes to the replacement of the dead cardiomyocytes with fibrosis, scar tissue formation, and modulation of cardiomyocyte structure and function (Nagaraju et al. 2019). Therefore, suppressing their activation (Meng et al. 2018) or involving them in neomyogenesis via direct reprogramming strategy may be exploited to add them into the repair process.

Lentiviral vectors have been extensively used for transgene delivery into various cell types, including the poorly dividing cardiomyocytes, owing to the stable, heritable gene transfection in both dividing and nondividing cells without inflammatory/immune response (Kim et al. 2001; Zhao et al. 2002). These advantages render them undeniably more appealing as transgene delivery vectors as compared to the other vectors. Attempts are underway to optimize protocols to generate enhanced viral vector titter to achieve transfection efficiency (He et al. 2021). However, one of the limitations of lentiviral vectors is their lack of site-specific uptake when delivered systemically, thus delivering the transgene to nontarget cells and tissues. Hence, the lentiviral vector is being combined with other strategies to increase its transduction efficiency.

In recent years, CMBs and MMBs (magnetic microbubbles)-hybrids with lentiviral vector combined with UTMD has gained popularity for efficient therapeutic gene delivery as UTMD causes increased sonoporation of the cells at the target

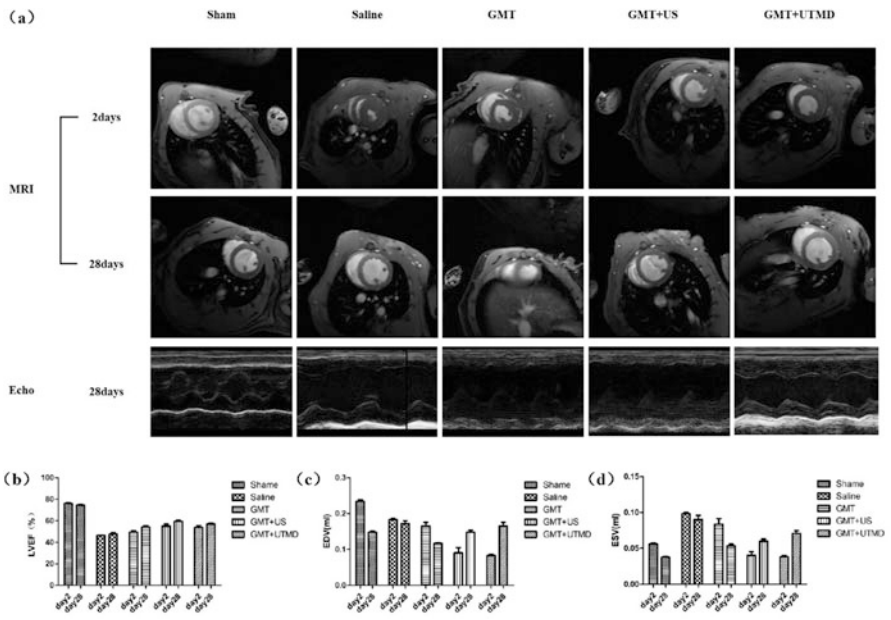

Fig. 7 In vivo heart function assessment by the 7 T MRI system for small experimental animal models. (**a**) Panel 1: MRI showing a cardiac cross-section at 2 days after surgery; panel 2: MRI of a cardiac cross-section on 28 days post-surgery; panel 3: M-mode echocardiography images depicting a cardiac cross-section on day 28 after surgery. (**b**) Quantitative analyses of LVEF on days 2 and 28 after surgery. (**c**) Quantitative analyses of LVEDV at 2 and 28 days post-surgery. (**d**) Quantitative analyses of LVESV on days 2 and 28 after surgery. (LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume)

sites to facilitate the genetic material uptake (Yang et al. 2011, Cool et al. 2013). These data successfully demonstrated the applicability of combining lentiviral vectors with CMBs, which allowed the viral vector's high binding capacity to achieve high gene transfection efficiency. In both the in vitro and in vivo experiments, direct-reprogramming GMT factors proved capable of cardiac repair. With UTMD, viral vectors were directly deposited in cardiac tissue and successfully repaired the infarcted myocardium. The immunofluorescence assay and histological examination confirmed neomyocytes and neo-angiogenesis during a 4-week follow-up in the rat model of MI. All the treatment groups showed significant LV-function improvement as compared with the control treatment group.

CMB–Lentivirus Hybrids in Gene Delivery

These data describe the synthesis of CMBs from DPPC, DPPE, and DOPE (Fig. 2a). The lentivirus–CMBs conjugation was confirmed visually by green fluorescence (Fig. 2c). It is important to mention that the characteristics of the synthesized

microbubbles, including their lipid composition, size distribution, residual charge, etc., significantly impact their genetic material loading capacity. Similarly, CMBs synthesis protocols involve the use of different types of cationic lipids integrated onto the lipid microbubble shell (Cao et al. 2017a; Negishi et al. 2016; Yang et al. 2018). It is pertinent to mention that the chemical composition of the cationic head groups are the significant determinants of the physiochemical properties of the CMBs besides remaining as the major determinants of their capacity to hybridize with the lentiviral vectors carrying the transcription factors of interest for delivery. Similarly, the inclusion of helper lipids contributes to the physiochemical properties as well as their transfection efficiency. As a result, the CMBs showed high loading capacity and transfection efficiency because of their charge coupling. In these experiments, the prepared CMBs yielded a mean zeta potential of 28.3 ± 1.4 mV. This cationic lipid has turned out to be an efficient and popular method for gene transfection and drug delivery. Because of the electrostatic adhesion between CMBs and anionic lentivirus particles, CMB–lentivirus hybrids could be a promising noninvasive platform for direct reprogramming in rats.

Advantages of Lentiviral Vectors for Direct Reprogramming

Besides their ability to transduce both dividing and nondividing cells with equal efficiency and low immunogenicity, the third-generation lentivirus packaging system involves several safety measures (Gill and Denham 2020). The virus-packaging system relies on four separate plasmids to produce lentiviral vectors; this arrangement decreases the chance of wild-type virus recombination events (Lukashev and Zamyatnin 2016). Moreover, the third-generation lentivirus packaging system deletes the crucial viral transcription activator gene *tat* and its regulatory region, thus generating self-inactivating (SIN) vectors (Sharon and Kamen 2018). Additionally, this system separates viral packaging genes, including *gag, pol*, and *rev* on two plasmids (Stellberger et al. 2017). Considering other features of this lentiviral vector system, including the deletion of accessory genes *vif*, *vpr*, *vpu*, and *nef*, which are vital for the pathogenesis of the virus, and replacement of the native HIV-1 envelope protein by heterologous envelope protein VSV-G, the third-generation lentivirus system maximizes the biosafety for its applications (Shearer and Saunders 2015).

For the production of the vectors, the lentiviral vector transfer plasmid for GMT overexpression and three plasmids containing packaging and envelope genes (pRsv-REV, pMDlg-pRRE, and pMD2G) were transfected into a HEK293T producer cell line, which then assembled the virus and released it into the culture medium. By restriction enzyme identification, PCR, and sequence analysis, the successful construction of the recombinant lentiviruses was confirmed, and there were no HIV-1 recombination events detected (Fig. 3). The lentiviral vectors were then concentrated to a high titer and tested for key characteristics before their use for direct cardiac reprogramming and UTMD application.

Improvement of Reprogramming Factors in Direct Cardiac Reprogramming

As discussed earlier, the CFs have a crucial role in the vicious cycle of LV remodeling post-MI. The adult functioning cardiomyocytes undergoing apoptosis and necrosis in the infarcted myocardium are replaced by fibrotic scar tissue as a part of the intrinsic repair process. Given the pivotal role of CFs in both reactive and reparative fibrotic activity (Hara et al. 2017), the strategy to involve them in neomyogenic repair of the infarcted myocardium has gained momentum that is based on their direct reprogramming into iCMs to restore cardiac function (Fu and Srivastava 2015). Although several combinations of transcription factors with or without microRNA manipulations, the published data so far provides mounting evidence confirming that GMT is an effective combination of cardiac-specific direct reprogramming factors both in vitro or in vivo for the generation of iCMs from CMs (Sadahiro et al. 2015; Jayawardena et al. 2015; Boon and Dimmeler 2015; Sinagra and Fabris 2016).

Tronolab lentiviral production system was successfully used to generate reprogramming lentivirus, and GMT viral titers achieved were 3.05×10^8, 3.25×10^8, and 3.02×10^8 TU/ml. During the transfection protocol in the experiment, 8 μg/ml polybrene was added to neutralize the electrostatic interaction between the cells and the viral particles that significantly contributed to improving the transfection efficacy. The successfully reprogrammed CFs yielded high expression of GATA4, MEF2C, TBX5, besides the expression of cardiac-specific cTnT (an important cardiac marker) at 48 h after sequential transfection with the GMT virus (Fig. 3). In vitro experimental data strongly support that the GMT lentivirus can directly and efficiently reprogram CFs, which is in agreement with other published studies (Fu et al. 2013; Inagawa and Ieda 2013; Song et al. 2012; Ieda et al. 2010). However, the success of direct reprogramming and its efficiency is determined by the proper reprogramming factors selection and well-cultured fibroblasts (Adams et al. 2021).

Besides the triad of GMT, several other combinations of cardiac-specific transcription factors (López-Muneta et al. 2020) and strategies can directly reprogram mouse and human fibroblasts into iCMs (Hashimot et al. 2016). Given the pivotal role of miRNAs in diverse signaling pathways regulating the cellular functions (Haider et al. 2015), they are being used for stem cell-based therapy to promote various functions, i.e., stem cell survival, differentiation, and to modulate their paracrine behavior, etc. (Haider et al. 2009; Kim et al. 2009; Haider et al. 2010; Kim et al. 2012a, b; Lai et al. 2012). They are also being used in various direct cardiomyogenic reprogramming protocols. For example, cocktails including GMT plus Hand2 (GHMT), and transient expression of four microRNAs, i.e., miR-1, miR-133, miR-208, and miR-499 (together termed as miRcombo), and chemical enhancement of direct reprogramming; therefore, further exploration for the optimal combination of reprogramming factors is still being pursued (Nam et al. 2014; Tani et al. 2018; Mohamed et al. 2017). The presence of miRNAs increased the rate of cardiomyogenic reprogramming and needed a shorter reprogramming period (Alfar

et al. 2018). Paoletti et al. used transient expression of miRcombo to directly reprogram human adult cardiac fibroblasts to develop iCMs (Paoletti et al. 2020). They have reported up to 11% of fibroblasts to adopt cardiac phenotype by day 15 after manipulation, as evaluated by flow cytometry for cTnT expression. And by day 30, the transdifferentiated cells exhibited spontaneous calcium transient. Manipulating these cocktails of reprogramming factors can increase the efficiency of direct cardiomyogenic reprogramming to generate iCMs for cell-based therapy or other potential theranostic applications (Chen et al. 2017; Ghiroldi et al. 2017).

Ultrasound-Mediated Localized Direct Reprogramming via Lentivirus Delivery for Cardiac Repair

With the recent developments in cardiac regenerative medicine, especially where stem cell therapy is being combined with gene delivery for a combinatorial approach, gene transfer has become a valuable tool in therapeutic research (Wu and Li 2017). The cells are genetically modulated to overexpress the gene/s of interest for delivery to the heart. The established delivery systems, including intramyocardial injection and coronary perfusion, are based on invasive procedures, which hinder their further clinical translation (Ni et al. 2016; Suzuki et al. 2011; Ebrahimi 2017).

The UTMD strategy is safe, noninvasive, and ensures high rate of organ-specific gene delivery. Several researchers have demonstrated the suitability of UTMD combined with in vivo viral vector transduction after intravenous administration in experimental animal models (Robertson 2016). By loading adenoviral vectors onto the surface of albumin microbubbles, investigators have proven the targeted gene expression in the heart facilitated by UTMD (Qian et al. 2018, Liao et al. 2014, Ma et al. 2015, Sun et al. 2013). Although these studies suggest that MCAs could be used in synergy with viral vectors to enhance a virus-based gene delivery to the myocardium, they did not show significant therapeutic effects of this approach. On the contrary, the abovementioned experiments successfully used CMBs loaded-GMT lentiviral vectors capable of directly reprogramming the CFs. With ultrasound at the resonance frequency (1.5 MHz), destruction of the microbubbles is induced in the heart, thereby ensuring targeted delivery of viral vectors. The newly formed cardiomyocytes were visualized by immunofluorescence specific for PHH3 in the infarcted area in all the treatment groups of animals (Fig. 4). After 4 weeks of follow-up, the treated groups showed a significant decrease in the total area of fibrosis and increased vascular density in the peri-infarct region (Fig. 5).

Cardiac MRI revealed significantly higher mean change in LVEF for each rat from the time of lentivirus administration until 4 weeks in the treated groups (Fig. 7). From the in vivo experiment, it can be concluded that the novel strategy of UTMD can target direct-reprogramming lentiviral vectors into the rat heart. In comparison with direct intramyocardial injection or in situ microbubble destruction, the therapeutic effect of intravenous administration of viral vectors by UTMD as a noninvasive technique was not significantly different. The microbubble–virus hybrids seem to be an efficient platform for direct reprogramming and may be suitable for translation into a potential clinical strategy for patients with end-stage heart disease.

Although the lentiviral vectors are useful tools for long-term gene transfer into the target cells and are extensively used in cell reprogramming research, the critical problem of this gene delivery strategy is its toxicity to unrelated organs, especially due to hepatic uptake (McCarron et al. 2016; Sirsi and Borden 2012). MCAs, however, can increase the therapeutic index and thus may reduce the toxicity of viral vectors to some extent. Histological examination of lungs, the liver, kidneys, and spleen was performed after H&E staining, which detected no damage or inflammation in these organs, thus revealing the safety of organ-specific technique to avoid damage to the unrelated/nontarget organs (Fig. 6).

Conclusion and Future Perspective

Direct reprogramming possesses several theoretical advantages, resolving the main challenges and issues associated with cell therapies. The approach has come a long way to define specific factors and cues needed to help somatic cells cross lineage restrictions and undergo transdifferentiation to adopt the phenotype of interest (Wang et al. 2021). The most significant feature of direct reprogramming is that the transdifferentiating somatic cells are guided to avoid intermediary pluripotency states and new phenotype. Because the lentiviral-based gene delivery is gaining popularity and acceptability for clinical applications due to of their superior characteristics (Milone and O'Doherty 2018), methods to generate integration-free iCMs are necessary and widely sought. This study implements the strategy of delivery of direct-reprogramming lentiviral vectors encoding for the triad of cardiac transcription factors-CMBs hybrid to a target infarcted myocardium by UTMD serving as a viable myocardial tissue repair therapy. Systematic in vitro and in vivo results have confirmed that administration of GMT induces post-infarct ventricular functional improvement, whereas UTMD enhances the efficacy. Based on this strategy, lentiviral CMBs are likely to be a safe and effective platform for targeted delivery of reprogramming factors for myocardial repair via neomyogenic differentiation of non-myocytes, i.e., CFs, in the infarcted myocardium. Furthermore, the strategy will not only help recover the lost myocytes during infarction episodes; it will also curtail CFs availability for cardiac fibrosis and fibrotic scar tissue formation. In summary, the combinatorial approach using lentiviral-CMBs hybrid together with UTMD may be developed as a potential therapeutic modality in the future for myocardial repair and regeneration.

A Summary of the Experimental Methods and Design

Vectors and Cells

Transcription factor genes *Gata4* (GenBank accession No. NM_144730.1), *Mef2c* (GenBank accession No. XM_006231731.2), and *Tbx5* (GenBank accession No. NM_001009964.1) were synthesized based on sequences available in the NCBI database and were cloned into plasmid pUC57. Amplified sequences were

then cloned separately into plasmids pCDH-MSCV-MCS-EF1-copGFP, Plvx-Puro, and pLVX-IRES. These plasmids were used to generate lentiviral vectors by means of the Tronolab lentiviral production system (Shanghai Telebio Co., Ltd.), which consisted of pRsv-REV, pMDlg-pRRE, and pMD2G. The packaging plasmids were co-transfected into the 293 T human embryonic kidney cell line (Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) to produce the lentiviral vectors encoding for respective transcription factor. The control lentivirus vector was constructed using a homologous lentiviral vector encoding an *eGFP* cassette and was also generated by means of the lentiviral-packaging plasmids in 293 T cells. The collected supernatants containing the virus at 48 or 72 h after transfection were pelleted by centrifugation and then filtered with a 0.45 μm pore size syringe. The generated lentiviruses were further concentrated for high titer and preserved in an −80 °C freezer.

Rat CFs harvested from a Sprague–Dawley adult male rat (iCells Biological Engineering Co., Ltd., China) were cultured in Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM/F12, Thermo Fisher, USA) supplemented with 15% of fetal bovine serum and 1% of a penicillin and streptomycin solution. The rats CFs were cultured in a humidified incubator under specific conditions (37 °C, 5% CO_2, 21% O_2, and 74% N_2).

Creation of CMBs and Microbubble–Virus Hybrids

The CMBs were fabricated via the typical emulsification–vibration process. Briefly, DPPC, DPPE, and DOPE were dissolved in chloroform uniformly and then redissolved in saline (10 ml) after rotary evaporation. Glycerine (500 μl) was added at 70 °C and emulsified for 2 h in a water bath. The obtained emulsion (1 ml) was transferred into a glass bottle (5 ml), for sulfur hexafluoride injection treatment. The emulsion and gas were integrated after strong vibration via CMBs. CMBs was characterized for concentration, size distribution, and zeta potential on a Zetasizer NANO ZS system.

Based on the electrostatic adsorption of the cationic CMBs and the anionic lentivirus, immunostaining of loaded and unloaded microbubbles was performed to examine the binding efficiency. The initial mean microbubble count in this experiment was 10^7. The virus/microbubble ratio was maintained at 1000:1. Before injection, virus/microbubble hybrid size and concentration were measured to ensure the proper characteristics (Nikon, Germany). To confirm the successful conjugation of the lentivirus and CMBs, 100 μl of a fluorescein isothiocyanate (FITC)-conjugated mouse antihuman p24 antibody (catalogue #ab20569, Abcam; dilution 1:50) was added to 200 μl of microbubbles in 1.0 ml PBS and mixed for 30 min at 4 °C. After that, the upper layer was used for fluorescence microscopy. Microbubbles loaded with the lentivirus were analyzed to determine whether the microbubble surface emitted strong fluorescence to prove the attachment of the viral vector, using microbubbles without lentivirus as a control.

To further demonstrate the efficiency of lentivirus hybridized with CMBs and mediated by UTMD, the experiment included the following groups: GFP-lentivirus alone, GFP-lentivirus with ultrasound exposure, and GFP-lentivirus loaded by CMBs with ultrasound exposure. Exposed to ultrasound at diverse power intensities, those CFs transfected by CMB–lentivirus complexes were further tested for appropriate intensity. The optimum conditions were identified via the evaluation of different settings, including the duty cycle of 1.5 W/cm^2, power of 1.5 MHz, and 15, 30, or 60 s duration. The cells were then seeded in 6-well culture plates for 48-hour incubation and were analyzed for fluorescence signals using a microscope.

Direct Reprogramming of CFs In Vitro

GATA4, MEF2, and Tbx5 vectors (3.05×10^8, 3.25×10^8, and 3.02×10^8 TU/ml, respectively) or the GFP control lentivirus vector (10^8 TU/ml) were added into the rat CF culture medium (DMEM/F12 plus 10% FBS8 and μg/ml polybrene) for 48-hour incubation. Under routine culture conditions, the cells were allowed to undergo transdifferentiation for 14 days after removing the medium. Later, the cells were collected for analysis.

A Western Blot Analysis

Reprogrammed cells (7 or 14 days after transfection) were extracted for total protein samples with RIPA buffer containing phosphatase inhibitors and protease. Using the Bradford protein assay kit (Bio-Rad, USA) and BSA as a protein standard, protein concentrations were determined for further experiments. The protein samples were electrophoresed on SDS-PAGE in a 10% gel, blotted onto membranes, and incubated with various antibodies: anti-Gata4 (Abcam, cat. # 84593), anti-Mef2c (ImmunoWay, cat. # YT2702), anti-Tbx5 (Proteintech, cat. # 13178-1-AP), anti–cardiac troponin T (anti-cTnT; ImmunoWay, cat. # YT5362), and anti–β-actin as an internal control (Abcam, cat. # 8226). A horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Abcam, cat. # 205718) was used as the secondary antibody. The quantitative results of bands density were calculated using ImageJ software.

Development of Experimental Rat Model of MI

Male Sprague–Dawley rats (weight 200–220 g) were purchased from the Department of Laboratory Animal Science, Shanghai Medical College, Fudan University, P.R. China. All animal experiments were performed according to the policy of Fudan University of Health (including its guide for the care and use of laboratory animals).

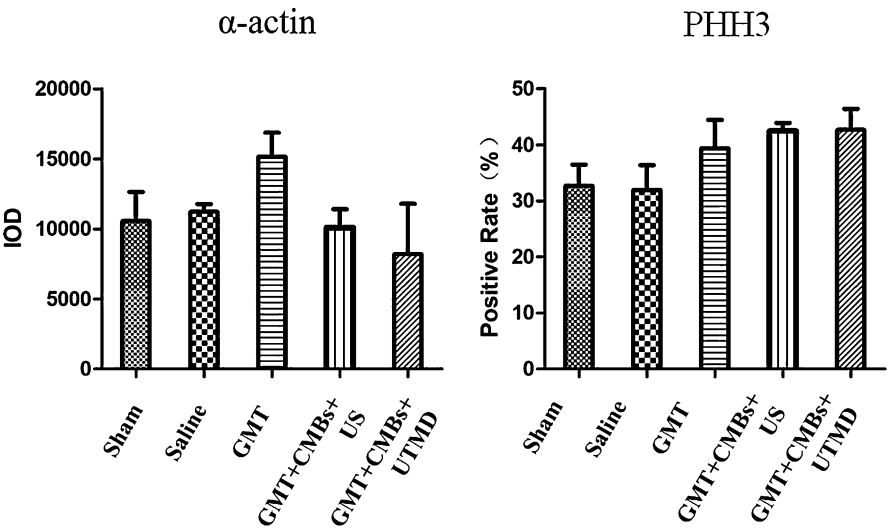

Figure S5 Statistics results of α-actin and PHH3 immunofluorescence for quantification of reprogrammed cells. (**a**) Statistics results of α-actin immunofluorescence (red) in peri-infarct area. (**b**) Statistics results of PHH3 immunofluorescence (green) positive rate in peri-infarct area

The rats were anesthetized with 10% chloral hydrate (0.3 ml per 100 g, administered intraperitoneally), intubated, and ventilated with an animal ventilator (Chengdu Instrument Factory, China). After ligation of left anterior descending (LAD) coronary artery with an 8/0 suture, elevated S-T segment and a Q-wave appearance on ECG confirmed the success of MI induction (Fig. S5). After the surgical procedure, 40 rats were randomly assigned into 5 experimental groups (n = 8 animals/per group): (i) Sham (opened/closed chest without ligation, untreated), (ii) Saline (rat chest opened with ligation and injection with 0.9% saline in the infarcted area, untreated), (iii) GMT lentivirus alone (MI-GMT), (iv) CMB/GMT lentivirus with ultrasound (MI-CMB/GMT + US), and (v) GMT lentivirus with CMBs injected via a tube inserted into the right internal jugular vein and ultrasound (MI-CMB/GMT + UTMD).

Delivery of Microbubble–Lentivirus Hybrids Using UTMD In Vivo

At a virus/microbubble ratio of 1000:1, lentiviral vector was incubated with CMBs at 37 °C. After 30 min, the mixture was diluted with saline to 0.5/rat (Zhang et al. 2017; Muller et al. 2008; Delalande et al. 2017). Ten minutes after LAD ligation surgery, 0.5 ml CMB–GMT lentivirus mixture was injected into the right internal jugular vein in the UTMD rat groups. Simultaneously, an ultrasound probe administered by a sonoporator (Chattanooga Group) delivered transthoracic ultrasound beam at frequency of 1.5 MHz for 5 min, power 1.5 W/cm^2, and probe diameter 1.2 cm.

Histological Examination

Rats were euthanized on day 28 after their respective treatment; the hearts removed for histological examination. Masson trichrome and hematoxylin-eosin (HE) staining were performed on 5 mm transverse sections from the entire left ventricle. The other organs removed, including kidneys, spleen, lungs, and liver, were also removed for histological examination. Immunohistochemical staining for CD31 (Abcam, cat. # 182981) and α-SMA (Abcam, cat. # 7817) was performed to evaluate angiogenesis in the peri-infarct area. Immunohistochemical staining for GATA4 (Abcam, cat# 84593), MEF2C (ImmunoWay, cat# YT2702), TBX5 (Proteintech, cat# 13178-1-AP), and cTnT (ImmunoWay, cat# YT5362) was also performed to evaluate in situ direct reprogramming in the peri-infarct area. We prepared cryo-sections of the peri-infarct area for phospho-histone H3 (PHH3, Abcam, cat. # 32107) expression to identify neomyocytes using fluorescence microscope fitted with a camera (Nikon, Japan). Optical overview images were captured using "scan large image" function of the microscope. Representative sections of the large image were next enlarged and evaluated after α-actin expression (Abcam, cat. # 179467). Both channels were later merged during image analysis.

Cardiac Magnetic Resonance Imaging (cMRI) and Echocardiography

Measurements were performed on a 7 T MRI system for small animals (Bruker, USA), provided by the Centre for Biomedical Imaging, Fudan University, China. According to the operating manual of Bruker Company, a series of four pilot transverse images and then a single slice (coronal and sagittal images) were acquired so that the software could get enough anatomical information for planning scans. A gradient cine sequence was employed to acquire contiguous short-axis slices from the apex to the base of the heart. Data analysis was performed in analytical software Segment v.1.8 to determine LV-functional parameters.

After inhalation anesthesia with 3% isoflurane, echocardiography was performed using a Vevo 2100 Imaging System (VisualSonics, Inc., Toronto, Ontario, Canada) provided by the Shanghai Institute of Cardiovascular Disease. Unaware of grouping and treatment, an investigator captured echocardiographic images of parasternal long-axis and short-axis views at specified points. M-mode tracings provided data on LVESV and LVEDV. The change in the LVEF after GMT administration was calculated for cardiac function assessment.

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Future Perspectives of Exosomal Payload of miRNAs in Lung Cancer

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Beatrice Aramini, Valentina Masciale, Giulia Grisendi, Federico Banchelli, Roberto D'Amico, Massimo Dominici, and Khawaja Husnain Haider

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B. Aramini (✉)
Division of Thoracic Surgery, Department of Experimental, Diagnostic and Specialty Medicine–DIMES of the Alma Mater Studiorum, University of Bologna, G.B. Morgagni–L. Pierantoni Hospital, Forlì, Italy
e-mail: beatrice.aramini@auslromagna.it

V. Masciale
Division of Thoracic Surgery, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy
e-mail: valentina.masciale@unimore.it

G. Grisendi · M. Dominici
Division of Oncology, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy
e-mail: giulia.grisendi@unimore.it; massimo.dominici@unimore.it

F. Banchelli · R. D'Amico
Center of Statistic, Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, Italy
e-mail: roberto.damico@unimore.it

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukairiyah, Saudi Arabia
e-mail: kh.haider@sr.edu.sa

K. H. Haider (ed.), *Handbook of Stem Cell Therapy*,
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Abstract

Exosomes are nanovesicles, which were first described in 1983 as a method by which cells disposed of their metabolic waste. Since the publication of these early reports, exosomes have emerged as important regulators of various processes and mechanisms that ensure the maintenance of cellular homeostasis. In cancer cells, exosomes have added roles in immunomodulation, metastasis, tumor growth and progression, chemo- and radioresistance, cell proliferation, and everything that puts the cancer cells in a position of advantage as compared to the normal cells. More recent studies have revealed that almost every cell type can release exosomes rich in the specific payload of bioactive molecules including miRNAs which are integral to intercellular communication via the transfer of the payload from the senders to the recipient cells. This chapter highlights the newly emerging function of exosomes as miRNA carriers in lung cancer and presents new perspectives for next-generation cancer treatments and targeted personalized medicine.

Keywords

Cancer-associated fibroblast · Cancer · Exosome · Lung cancer · Microvesicles · miRNA · Metastasis · Payload · Tumor

Abbreviations

Alix	Programmed cell death 6-interacting protein
BMP	Bis(monoacylglycerol) phosphate
EMT	Epithelial-to-mesenchymal transition
ESCRT	Endosomal sorting complexes required for transport
EVs	Extracellular vesicles
HIF	Hypoxia-inducible factor
hnRNP	Heterogeneous nuclear ribonucleoproteins
HPV	Human papillomavirus
HUVECs	Human umbilical vein endothelial cells
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MHC	Histocompatibility complex
miR	MicroRNA
miRNAs	MicroRNAs
MVBs	Multivesicular bodies
nSMase	Neural sphingomyelinase 2
PADC	Pancreatic adenocarcinoma
VEGF	Vascular endothelial growth factor

Introduction

Exosome Composition

The history of exosomes dates back to 1946, when it was discovered that plasma prevented blood clotting (Hargett and Bauer 2013). It was also demonstrated that the observed anticlotting activity was due to the presence of platelet-derived nano-sized vesicles. In 1983, researchers in the Johnstone laboratory reported an interaction between the reticulocyte transferrin receptors and nanovesicles derived from sheep reticulocytes. These multivesicular bodies (MVBs)-derived vesicles were named exosomes (Wolf 1967; Pan and Johnstone 1983; Pan et al. 1985). It was also observed that exosomes were reservoirs of RNA for transfer to the recipient cells as part of cell-to-cell communication. Since the publication of these data, various research groups have extensively studied and reported the complex composition of the exosomal payload that may include proteins, lipids, mRNA, miRNA, etc. (Mashouri et al. 2019). Given their ultramicroscopic size, they are generally viewed by electron microscopy. Earlier after their discovery, exosomes were considered as a part of the cell's disposal system to get rid of metabolic waste. Subsequent research revealed that exosomes mirrored the MVBs in their constitution and some proteins usually associated with MVBs were also detectable in exosomes (Pettersen and Llorente 2018; Henne et al. 2013). These data also helped in the elucidation of the underlying mechanisms of their release, although some of these molecules have not been well-characterized due to the complexity of their structure.

Based on their size, structural features, and payload composition, exosomes are one of the subgroups of extracellular vesicles (EVs), which are secreted by the cells. Electron microscopy and in-depth proteomic, lipidomic, and genomic analysis have been used to characterize exosomes for their structure and molecular composition (D'Asti et al. 2012). They are the intraluminally generated, and their composition shows a close similarity with the parent cells of their origin. Typical constituent proteins of exosomes include the adhesion molecules, the major histocompatibility complex (MHC) class I and class II, and the transferrin receptors. Additionally, some nonspecific exosomal proteins include Rab2, Rab7, flotillin, annexin, heat shock proteins, cytoskeleton proteins, and the programmed cell death 6-interacting protein (Alix), which mediate MVB formation (Mathivanan et al. 2010). The lipid components are cell-specific and play a major role to protect the exosomes and maintain a correct balance in the recipient cells (György et al. 2011; Azmi et al. 2013). Lipids such as lysobisphosphatidic acid in MVBs generate intraluminal vesicles, i.e., exosomes (Mashouri et al. 2019). The interaction between lysobisphosphatidic acid and Alix promotes the inward generation of the MVB membrane (D'Asti et al. 2012). Sphingomyelin, phosphatidylcholine, and bis(monoacylglycerol) phosphate (BMP) contribute to differentiate the vesicles from each other. In particular, BMP, a negatively charged protein, is specific for endosomes, and it is involved in exosome formation (Akgoc et al. 2015). Recent researches defined exosomes as

microparticles that can influence cellular homeostasis by changing the lipid profile of the recipient cells. ExoCarta and Vesiclepedia are two of most comprehensive databases about exosomes characteristics registering thousands of entries for proteins, mRNAs, and lipids, and they are useful for exosomes' molecular definition (Pathan et al. 2019; Mashouri et al. 2019).

Biological Functions of Exosomes

Exosomes show active participation in intercellular communications which has been ascribed to several physiologic and pathologic processes (Meldolesi 2018). Their genesis is generally classified as an ESCRT-dependent or ESCRT-independent mechanism; however, the pathways might not be entirely discrete (Zhang et al. 2019). Rather, these two mechanisms work in harmony, while the resultant diverging subpopulations of exosomes originate by the different contributions from ESCRT-dependent and ESCRT-independent process machinery involved therein (Valadi et al. 2007; Andre-Gregoire and Gavard 2016). Although the complete mechanism involved in the formation of MVBs proceeds in a controlled fashion but remains less well understood, it is believed that a late endosome infolding occurs to form intraluminal vesicles before becoming an MVB. It has been described that the process of exosomal membrane invagination is aided by a well-orchestrated and controlled interaction between syndecan-syntenin and Alix protein (Tkach and Thery 2016; Simons and Raposo 2009). It is suggested that subpopulations of MVBs product depend upon the cell type, while others have proposed that the various mechanisms may take place in the same cell (Pitt et al. 2016). It is pertinent to mention that the release of exosomes is an important feature of several cell types and that too in terms of their release into various body fluids carrying a specific payload composition that is influenced by the culture conditions.

Despite the scarcity of information regarding the biological and therapeutic benefits from in vivo experimental animal models and limited in vitro characterization data, the actual problem is related to the purification protocols based on the ultracentrifugation technique which is an inefficient method for exosomes purification and quantification because exosome may get recaptured by the cells. The method of purification fails to ensure fool-proof complete recovery of the exosomes from the conditioned medium from the in vitro cultured cells or from the biological fluids. The inefficiency of the isolation and purification protocols creates a discrepancy between the secreted amount of exosomes and their biological activity.

Exosomes also carry rich amounts of immunosuppressive molecules. For example, the placenta-derived vesicles isolated from the blood of pregnant women are rich in ligands for natural killer cells. Exosomes have also been found in mice bronchoalveolar lavage fluid which may carry tolerizing molecules from a specific allergen-tolerized animal and release of proinflammatory cytokines in the airway epithelium (Prado et al. 2008). Yang et al. have ascribed the tumorigenic potential of lavage fluid to its exosomal contents during in vitro as well as in vivo studies (Yang et al. 2019). Similarly, the release of exosomes by the eukaryotic parasites or

pathogens significantly contributes to the host's tolerance by quenching the immune response or promoting reactions against the pathogen. More recent studies have shown that tumor-derived exosomes are rich in immunosuppressive molecules, which help the cells to evade host immune response (Olejarz et al. 2020). In addition to the immune-regulatory properties, exosomes also participate in tissue repair. For example, mesenchymal stem cells (MSCs)-derived exosomes effectively participate in myocardial repair (Haider and Aramini 2020). They also transfer pathogenic proteins such as prions and amyloid peptides besides others with as yet less-well-characterized functions (Bellingham et al. 2012; Bissig et al. 2013).

The normal healthy cells also secrete exosomes to transfer their payload to other recipient cells as an integral part of intercellular communication. A payload analysis of exosomes reveals that a typical exosome contains, besides other components, a panel of miRNAs, although the mechanism of miRNA selection to be part of the select miRNA panel has not been clarified yet. Moreover, it is also not known whether the members of the select panel of miRNAs cooperate, synergize, or inhibit each other to achieve a balance in their functioning. These mechanisms have been discussed in-depth in numerous previously published researches (Schwarzenbach and Gahan 2019; Urbanelli et al. 2013; Hammond 2015; Simons and Raposo 2009). The off-loading of the exosomal payload and its uptake by the recipient cells primarily occurs by endocytosis that facilitates the formation of a vesicular early endosome, which can be recycled in the cell by the cell machinery, or it is passaged into the nucleus (Tkach and Thery 2016). At this level, the function of the vesicle is similar to a lysosome.

Exosomes and Cancer

Similar to the other areas of research, the application of extracellular vesicles is gaining popularity in the field of cancer research as they provide interesting novel targets for future anticancer diagnostics and therapies (Pitt et al. 2016; Keller et al. 2011). The factors released by exosomes contribute significantly toward the formation, progression, dissemination, and recurrence of tumors. Moreover, the exosomal payload also reduces their responsiveness to the medical treatments due to a specific cell-to-cell communication with the tumor microenvironment (Pitt et al. 2016; Keller et al. 2011). It is pertinent to mention that cell-to-cell communication is fundamental to various physiological and pathological processes both during embryonic development and in the postnatal life to ensure cellular homeostasis and reparability (Chargaff and West 1946; Meldolesi 2018). From among the various possible mechanisms responsible for the transfer of biomaterials between the cells, cell-to-cell contact is important for the nearby cells in each other's vicinity, while the involvement of body fluids to carry vesicular payload is important for long-distance communication (Sung et al. 2015). Luga et al. have shown that in orthotropic breast cancer model in mice, concomitant injection of breast cancer cells and fibroblasts increased the rate of metastasis, which showed dependence on Wnt-planar cell polarity in the breast cancer cells and an exosomal component of CD81 in the fibroblasts (Luga et al. 2012). The authors concluded that the exosomal activity of

fibroblasts altered breast cancer cell behavior. Similarly, the role of tumor-associated exosomes in contributing integrin has been elucidated that enhanced tumor progression (Paolillo and Schinelli 2017). The malignant cell-derived exosomes alter the tumor microenvironment such that it becomes conducive for the development and progression of the tumor via interference with the cell adhesion mechanisms involving integrins and integrin ligands (Maia et al. 2018; Zheng et al. 2018). In particular, exosomal integrins are involved throughout, from colonization by cancer cells to the composition of a niche to facilitate metastasis (Richards et al. 2017).

Exosomal miR-105 facilitates the dissemination of the tumor cells by acting on the tight junction, thus compromising the integrity-endothelial cell barrier and weakening its functioning as a barrier (Zhang et al. 2018a, b). Exosomes also interfere with the permeability of the blood vessels. In colorectal cancer cells, miR-25-3p regulates VEGF-receptor 2, tight junction protein, and claudin-5 in endothelial cells. Exosomes secreted by the tumor cells also cause differentiation of the cells constituting their microenvironment by remodeling the extracellular matrix (Syn et al. 2016). Besides miRNAs, tumor cell-derived exosomes also contain a complete panel of bioactive molecules including HIF, TGF-β, caveolin, and b-catenin which is supportive in epithelial-to-mesenchymal transition and contributes to premetastatic niche formation (Syn et al. 2016; Becker et al. 2016). Pancreatic adenocarcinoma (PADC) is rich in cancer-associated fibroblasts which are inherently resistant to gemcitabine and promote survival of cancer cells (Richards et al. 2017). While elucidating the underlying mechanism, it was observed that cancer-associated fibroblasts-derived exosomes were responsible for this cytoprotective and proproliferative activity of PADCs in the presence of gemcitabine. Molecular studies revealed that cancer-associated fibroblasts-derived exosomes were able to transfer Snail and its target miR-146a to the recipient cancer cells to impart cytoprotective and cell-cycling activity in the recipient cells.

Interestingly, abrogation of the exosome release from cancer-associated fibroblasts also abrogated the cytoprotective effects on the cocultured cancer cells in the presence of gemcitabine. Cancer-associated fibroblasts-derived exosomes also have a role in glycolysis via increasing the absorption of glucose, which is considered as a contributing factor in the ongoing growth of cancer even under unfavorable reduced nutrition and hypoxic condition (Richards et al. 2017). In another study, it was observed that PADCs with high metastatic potential could release exosomes rich in migration-inhibitory factor, which supported premetastatic niche formation in the liver (Costa-Silva et al. 2015). The abrogation of emigrational inhibitory factor also abolished metastatic niche formation as well as metastasis PADCs in the liver.

Angiogenesis is indispensable for tumor development, progression, and dissemination (Katoh 2013; Zuazo-Gaztelu and Oriol 2018). In this regard, tumor cell-derived exosomes contribute significantly to the formation of neovasculature and increase in vascular density via activation of VEGF signaling (Nishida et al. 2006; Gluszko et al. 2019). Besides VEGF, the angiogenic activity in the tumor tissue is supported by various other proangiogenic molecules, i.e., basic fibroblast growth factors, platelet-derived growth factor, etc., which are also delivered by the tumor-derived exosomes TDEs (Sharghi-Namini et al. 2014; Monteforte et al. 2017).

Ludwig et al. have evaluated the role of tumor cell-derived exosomes in pro-angiogenic response during in vitro assays on HUVECs. The exosomes were derived from PCI-13 (HPV−) and UMSCC47 (HPV+) cell lines and the plasma of head and neck squamous cell carcinoma patients and were labeled before use in angiogenic assays (Ludwig et al. 2018). The exosomes were taken up by HUVECs in the culture within 4 h after treatment and stimulated HUVEC proliferation, migration, and tubulogenesis. Similar data were obtained after the treatment of experimental mice model of oral carcinoma after intravenous exosome treatment. On the same note, profiling of the glioblastoma-derived exosomes revealed high levels of miR-221, proteoglycans, glypican-1, and syndecan-4 that was ascribed to the acutely angiogenic nature of glioblastoma (Hoshino et al. 2015). Tumor cell-derived exosomes containing metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which is associated with angiogenesis and metastasis in epithelial ovarian cancer, induced proangiogenesis gene expression in HUVECs (Qiu et al. 2018). Put together, these data signify the importance of exosomes in angiogenesis and support their future use as targets for tumor cells by suppressing endothelial cell migration, their phenotype alteration, and vascular sprouting (Harding et al. 2013).

The Pathways of Exosomal miRNA Secretion

The cancer cells-microenvironment interaction essentially occurs through the release of bioactive molecules which contributes significantly toward the development and progression of tumors (Ungefroren et al. 2011; Kohlhapp et al. 2015). Despite the cell-specific role of miRNAs in tumor suppression and drug resistance, the participation of miRNAs in non-cell-autonomous mechanisms remains less explored. Although knowledge about the secretory RNA has been around since long, however, only recent reports have shown that miRNAs, which control various cellular processes through the regulation of specific multiple gene targets, are secreted by cells as part of the paracrine activity of the cells in the form of exosomal payload (Yu et al. 2016; Sansone et al. 2017). These data signify the emergence of both miRNAs and exosomes as the novel constituents of cells' paracrine activity with relevance to intercellular communication (Kalluri and LeBleu 2016). The role of neutral sphingomyelinase 2 (nSMase2) in the release mechanism of miRNAs and the functional activity of the released miRNAs in the recipient cells has been reported by Serrano-Heras et al. (2010). It is interesting to note that the tumor-suppressor miRNAs released by the normal cell potentially retain and impart gene silencing activity in the recipient tumor cells that may lead to abrogation of tumor cell growth (Huang et al. 2013). The critical role of exosomal miRNAs in intracellular communication, specifically between immune cells, endothelial cells, and cancer cells, is now becoming more obvious; however, their role in cancer metastasis remains less well established (Bhome et al. 2018).

Exosomal miR-210 transfer from metastatic cancer cells to the endothelial cell alters them to favorably support the process of cancer cell migration through neovascularization, thus helping in their metastatic spread. Molecular studies

revealed that miR-210 controlled the process of neovascularization by the suppression of its specific downstream target genes (Jung et al. 2017). The changes in the miRNA profile have been ascribed with different types of cancers wherein the expression of some miRNAs is either upregulated or downregulated (Esquela-Kerscher and Slack 2006; Girard et al. 2008; Peng and Croce 2016). On the same note, changes in the expression of miRNAs either support or inhibit the development and progression of cancer (Ota et al. 2004). The data supporting the critical involvement of miRNAs in cancer pathogenesis has led to their future use as potential therapeutic targets. Moreover, the huge amount of information regarding miRNA families that are altered in cancers will be important for the development of miRNA signatures which will be helpful in the diagnosis and therapy of cancers (Berindan-Neagoe et al. 2014).

The involvement of Argonaute proteins in miRNA function is well established. However, some exosomal miRNAs are Ago2-free (Arroyo et al. 2011). Instead, they are identified by specific proteins (e.g., the heterogeneous nuclear ribonucleoproteins hnRNPA2B1 and hnRNPA1) that selectively allow loading of miRNAs into exosomes (Janas et al. 2015). miRNAs loading into the exosomes is a well-orchestrated and regulated process. Villarroya-Beltri et al. have shown that miRNAs are loaded into exosomes via sumoylated hnRNPA2B1 (Villarroya-Beltri et al. 2013). Alternatively, the process involves two other heterogeneous nuclear ribonucleoproteins (hnRNP family proteins), namely, hnRNPA1 and hnRNPC. These proteins bind with exosomal miRNAs for their sorting process. The second mechanism is based on neural sphingomyelinase 2 (nSMase2) pathways (Kosaka et al. 2013), whereas the third mechanism is based on the 3′ ends of endogenous miRNAs. miRNAs are transported by RNA-binding proteins to the lipid raft-like region of the cytoplasmic leaflet of the MVB-limiting membrane, where miRNAs with the highest affinity are engaged (Janas et al. 2015). Once attached, the miRNA initiates a spontaneous invagination to produce intraluminal vesicles (Schwarzenbach and Gahan 2019).

Exosomal miRNAs in Cancer Progression

Several miRNAs are actively involved in various cellular processes (Yamakuchi et al. 2008; Pedroza-Torres et al. 2019; Chen et al. 2019). They are also involved in cancer progression and metastasis (Dilsiz 2020). Due to their critical roles and their presence in exosomes, miRNAs are considered to be promising markers for cancer diagnosis and prognosis (Wang et al. 2019). They can be used singly or in combination for cancer diagnosis (Tang et al. 2020). Cancer cell proliferation requires the dysregulated expression of cell cycle-related proteins. During the last decade, several cancer-secreted exosomal miRNAs have been identified as key regulators of cancer cell functioning. Some of the important ones from the long list include miR-200b, miR-21, miR-6869-5p, miR-9-3p, miR-let-7a, and miR-193a which work though diverse molecules such as p27, proinflammatory cytokines like IL-6 and tumor necrosis factor-α, FGF-5, and Caprin-1 (Teng et al. 2017; Zhang et al. 2018b; Liang et al. 2018; Tang et al. 2018; Liu et al. 2019).

Several studies have demonstrated that exosomal miRs also have a critical role in tumor cell apoptosis (Eichelser et al. 2014; Wei et al. 2016). For example, miR-128 decreases the expression of prosurvival protein Bcl-2 to enhance tumor cell survival. Eichelser et al. have reported analyses of the blood serum samples for free and exosomal miRNAs in 168 breast cancer patients. Exosomal miRNA profiling revealed significantly higher levels of miR-101, miR-372, and miR-373, while miR-373 was shown to interact with the estrogenic receptor for inhibition of the apoptosis cascade (Eichelser et al. 2014). The authors concluded that exosomal miR-373 was strongly linked with a more aggressive form of breast cancer. A recently published study has shown that conditioned medium derived from human adipose tissue-derived MSCs inhibited cell proliferation and activated cell apoptosis signaling in A2780 human ovarian cancer cell line (Reza et al. 2016). The authors attribute these actions of the conditioned medium with the presence of MSCs-derived exosomes, which upregulated proapoptotic signaling molecules including BAX, Casp3, and Casp9 with a concomitant decrease in antiapoptotic Bcl2. Exosomal analysis for miRNAs revealed two miRNAs, which were commonly expressed in three samples of the conditioned medium collected during independent experiments. These findings establish a clear interaction between MSCs and cancer cells via exosomal activity. miRNAs also participate in the relapse of the tumors. Exosomal miRNAs are regulators of EMT that contribute to the downregulation of E-cadherin and β-catenin and the expression of N-cadherin and vimentin markers which are specific to the mesenchymal phenotype. In particular, exosomal miR-21 can decrease E-cadherin expression, contributing to the induction of EMT-related changes in oral squamous cell carcinoma cells. Additionally, miR-1246 suppresses N-cadherin and vimentin activities and therefore interferes with the induction of the EMT process in prostate cancer cells (Bhagirath et al. 2018).

miRNAs participate in tumor-endothelial cells' crosstalk to regulate angiogenesis and cancer progression. For example, miR-103 targets VE-cadherin and P120-catenin, which are components of the endothelial cell junction to increase vascular permeability (Fang et al. 2018). Cells secreting exosomal miR-210 in hypoxic leukemia induce the endothelial cell activation to participate in the angiogenic cascade (Tadokoro et al. 2013). In miRNA profiling of chronic myeloid leukemia cell line LAMA84, 124 miRNAs were identified out of which miR-126 was highly overexpressed in the exosomes as compared with the cellular contents. Further experimentation with miR-126 revealed that it negatively regulated the motility of leukemia cells via altering CXCL12 and VCAM-1 expression levels (Taverna et al. 2014). Exosome profiling of the metastatic breast cancer cells revealed the extensive presence of proangiogenic miRNAs including hypoxamir-210, which is a known regulator of proangiogenic growth factor expression (Kosaka et al. 2013). The data was produced by in vitro culture of mouse cell lines 4 T1 and MCF7 breast cancer cell line, and the exosome isolation was carried out by ultracentrifugation and labeled with fluorescent tracking dye. The culture of HUVEC with exosomes significantly enhanced their activity during in vitro angiogenesis assays, which were more attributed to miR-210 with a significant role for neutral sphingomyelinase-2 for exosomal transfer of miRNAs. Similar observations have

also been reported with miR-23a from nasopharyngeal carcinoma cell-derived exosomes during in vitro angiogenesis assays which act by abrogation of its downstream target gene TSGA10 expression (Bao et al. 2018). On the other hand, exosomal miR-9 abrogated nasopharyngeal carcinoma cells' motility and angiogenesis via its downstream target gene midkine, thus interfering with Akt signaling (Lu et al. 2018).

Exosomal miRNAs also participate in the metastatic spread of cancer cells. In this regard, exosomal miR-148a is reported to cause proproliferative activity in cell glioblastoma by targeting its downstream gene CADM1 via STAT3 involvement (Cai et al. 2018). The authors also reported a negative correlation between miR148 and CADM1 gene expression profile in the patient samples. These data also highlight the possible use of exosomal miR-148a as a novel target for the development of future treatment strategies. Exosomal miR-423-5p is another important regulator of the cancer cell cycle through SUFU gene expression (Yang et al. 2018). In lung cancer, exosomal miR-96 has been shown to increase cancer cell proliferation and motility by targeting its downstream target gene LIM domain-only protein 7 (LMO7) (Wu et al. 2017). Some of the other important mediators of cancer cell activities reported thus far include miR-125a, miR-126, let-7b, miR-222, and miR-6126 (Felicetti et al. 2016; Kanlikilicer et al. 2016; Zhang et al. 2018b).

Tumor-Derived Exosomes and Lung Cancer

The function of tumor-derived exosomes in nonsmall cell lung cancer (NSCLC) implicates a combination of intricate mechanisms as summarized in Fig. 1.

One of the primary factors in cancer growth and progression in NSCLC is the rich presence of cancer-associated fibroblasts in the tumor microenvironment. These cancer-associated fibroblasts serve as a plentiful source of exosomes containing a payload of diverse molecules including RNA, lipids, amino acids, and tricarboxylic acid cycle intermediates (Zhao et al. 2016; Zheng et al. 2018). As discussed earlier, tumor-derived exosomes are instrumental in tumor angiogenesis, which facilitate tumor growth and metastasis. The induction of angiogenesis and tumor growth involves the TGF-β1-dependent pathway and stimulates fibroblasts differentiation (Webber et al. 2015). Hypoxic lung cancer cells cultured under low oxygen conditions released exosome which contained miR-23 for increased angiogenesis and enhanced permeability of the vessels through the targeting of tight junction proteins (Hsu et al. 2017). Exosomal miR-21 is regulated by the STAT3 pathway, which enhances the level of VEGF and consequently malignant transformation of the bronchial epithelium (Liu et al. 2016). Exosomal miR-210 is a hypoxamir, which regulates the levels of tyrosine receptor kinase A3, and induces vascular density in the tumors and supports the growth of the tumor cells (Cui et al. 2015).

Lung cancer cell-derived exosomes participate in lung cancer progression by interacting with the other constituent cells of the tumor microenvironment. Mesenchymal stem cells are transformed into a proinflammatory phenotype via the Toll-like receptor (TLR)-NFκB pathway by the lung cancer cell-derived exosomes. These

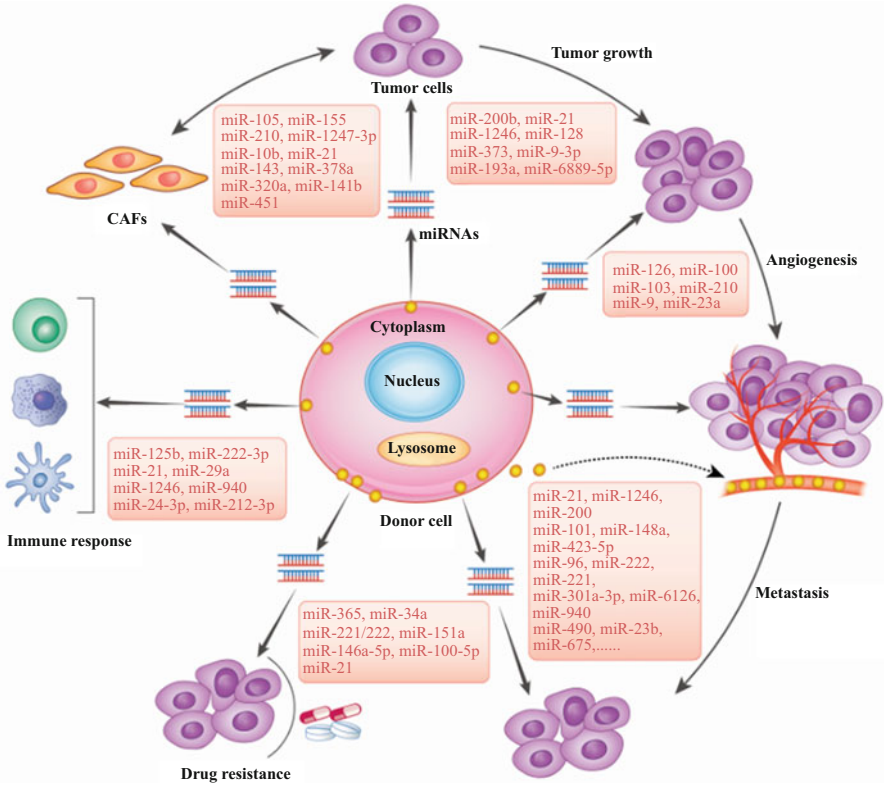

Fig. 1 Tumor-derived exosomal and their secreted miRNAs. Exosomal miRNAs disseminate information between cancer cells and immune cells (macrophages, T cells, and dendritic cells) and contribute toward the microenvironment to make it conducive for the cancer cells to thrive

exosomes facilitate tumor progression by interfering with the immune cell functioning to help the tumor cells escape the immune surveillance mechanism by reprogramming the immune cells (Ridder et al. 2015; Whiteside 2016). These studies highlight the role of exosomal payload in the growth and progression of NSCLC, which needs to be investigated further in future studies.

A major cause of death in lung cancer patients is the metastasis, which is multifactorial, regulated, and completed in several steps. However, only a few of the cancer cells can complete the entire process in a tissue-specific manner during which they preferentially lodge in different body tissue. For example, NSCLC cells preferentially disseminate and home into the brain, bone, and liver with a direct or indirect mechanistic role for tumor-derived exosomes (Wood et al. 2014). The main role of exosomes in the metastatic process is their contribution to the formation of lung cancer microenvironment and increasing the invasiveness of the tumor cells (Milane et al. 2015; Fujita et al. 2015). Some aspects of the tumor, including hypoxia, acidosis, and inflammation, can induce the tumor cells to release exosomes with their specific payload necessary to form the microenvironment with the

consequent growth of the tumor and its dissemination (King et al. 2012; Wang et al. 2016a). While elucidating the role of tumor cell-derived exosomes, Wang et al. showed that exosomal TGF-β and IL10 significantly contributed toward the emigrational activity of cancer cells (NCI-H1688 and NCI-H2228) during their experiment and demonstrated that exosomes derived from metastatic small cell lung cancer cell line NCI-H1688 were more effective in promoting lung cancer cell migration as compared to NSCLC cell line NCI-H1688-derived exosomes (Wang et al. 2016a). Hoshino et al. have shown development of specialized actin structures invadopodia which serve as exosome release and docking area. Abrogation of invadopodia significantly reduced exosome secretion during in vitro culture of the tumor cells, thus revealing a direct relationship between invadopodia formation and the rate of exosome release from the tumor cells (Hoshino et al. 2013). During an interesting in vitro study, human PCA LNCap and PC3 cells were subjected to either normoxic (21% oxygen) or hypoxic culture conditions (1% oxygen) to collect their secreted exosomes separately. The exosomes collected from the hypoxic cultured cells not only were smaller average size, but they also were more effective in promoting the motility of the naïve PCA LNCap and PC3 cells. The exosomes collected under hypoxic conditions also showed higher metalloproteinase activity besides being rich in various signaling molecules including IL6, tumor necrosis factor-α, TGF-β, Akt, and β-catenin (Ramteke et al. 2015). A recently published study has reported that exosomes released by colorectal cancer cell LM1215 triggered Wnt signaling in the coculture-recipient cells (Kalra et al. 2019). The exosomal transfer of mutant β-catenin to the nucleus of the recipient cells was confirmed by proteomic analysis.

Tumor-derived exosomes are also considered to be prognostic markers in NSCLC (Wang et al. 2016b). In a study involving 276 NSCLC, exosomes were isolated from the plasma samples and assessed for surface expression of various markers using an Extracellular Vesicle Array. From among the 49 antibodies which showed reactivity with surface proteins of the exosomes, although NY-ESO-1, placental alkaline phosphatase (PLAP), epidermal growth factor receptor (EGFR), Alix, and EpCam correlated well with overall survival, NY-ESO-1 was the only one which maintained a significant correlation in all the samples, thus signifying its prognostic value in NSCLC patients (Sandfeld-Paulsen et al. 2016). On the same note, Liu et al. have reported a significant association between exosomal miR-10b-5p, miR-23b-3p, and miR-21-5p with poor overall survival in NSCLC patients (Liu et al. 2017). On account of their specificity and easy to isolate from the body fluids which can be sampled noninvasively, exosomes may be novel biomarkers with diagnostic and prognostic value for tumor patients in the clinical perspective (Zhao et al. 2015).

Future Perspectives of Exosomal miRNAs in the Clinics

The molecular targets and the signaling involved therein as drug targets, and transporters to regulate cellular processes, i.e., cell survival and proliferation, have been shown with an integral regulatory involvement of several miRNAs (Corcoran et al. 2012). The payload of exosomes, despite having diverging composition even from the same cells under a different set of their microenvironment, is rich in proteins,

DNA, and RNA that is actively involved in the activation and inhibition of the pathways that are crucial during chemotherapy, radiotherapy, and targeted therapies. For example, it was recently described that miR-34a (with tumor-suppressor activity) abrogation during chemotherapy of prostate cancer treatment with paclitaxel was the primary cause of chemoresistance in the patients. Given its significant tumor-suppressor activity, miR-34a is now being considered as an established predictive biomarker of prostate cancer progression during paclitaxel-based chemotherapy via Bcl-2 gene expression regulation (Corcoran et al. 2014). Other researchers have highlighted the increase in tamoxifen sensitivity of breast cancer cells after the internalization of exosomes in tamoxifen-resistant breast cancer cells. This mechanism seems to be regulated by miR-221 or miR-222 delivered by the exosomes (Wei et al. 2014). Furthermore, in the lung cancer field, exosomal miR-21 has been investigated as a biomarker of the therapeutic outcome in NSCLC, and it was associated with acquired resistance to the treatment with EGFR and tyrosine kinase inhibitors (Li et al. 2014). Similarly, exosomal miR-21 is a crucial determinant of the radiosensitivity of tumor cells. Successful abrogation of miR-21 inhibits PI3K-Akt signaling to enhance their radiosensitivity (Ma et al. 2014). These data signify the role of miR-21 as an important predictor of response to the drug therapy and its worsening outcomes during chemotherapy (Ma et al. 2014; Schwarzenbach 2017). Hence, exosomal miRNAs could form the basis of personalized therapy on account of their potential as targets for therapy and their role in drug resistance.

Another important aspect of exosomal miRs is their potential role to serve as biomarkers (Schwarzenbach 2017). Several exosomal miRs isolated from tumor patients' plasma samples have the potential to serve as biomarkers as summarized in Fig. 2. For example, exosomal miR-21 is contained in plasma but has also been detected in fecal samples of the patients and therefore can be exploited as for colorectal cancer diagnosis (Rotelli et al. 2015). Other exosomal miRNAs that are upregulated in receptor-negative breast cancer patients have been recently reviewed and discussed in-depth (Yu et al. 2016; Stevic et al. 2018; Wu et al. 2020). The upregulated expression of miR-1290 and miR-375 has a negative prognostic value on account of its association with the poor overall survival in castration-resistant prostate cancer (Corcoran et al. 2012, 2014), while exosomal miR-19a is linked to the recurrence in colorectal cancer (Matsumura et al. 2015).

Exosom-derived miRs are also the inhibitors of tumor development which changes their use as part of future novel anticancer therapies. Nevertheless, the correlation and interaction between miRNAs, TDEs, and the immune system require further clarification. For example, the release of miR-142 and miR-223 post-transcriptionally regulates the expression of various proteins in hepatocarcinoma cells (Aucher et al. 2013). Similarly, exosomal miR-29c has antiapoptotic activity due to the suppression of Bcl2 and MCl-1 expression that leads to preventing bladder cancer cells' apoptosis (Xu et al. 2014). Moreover, exosomal miR-127 and miR-197 increase the rate of cell proliferation and promote the metastatic transfer of tumor cells. Hence, these exosomal miRNAs can reduce the effectiveness of tumor treatment by promoting resistance to therapies (Lim et al. 2011).

The manipulation of these miRNAs may significantly contribute to the development of novel cancer treatments, especially in patients with metastatic disease. One

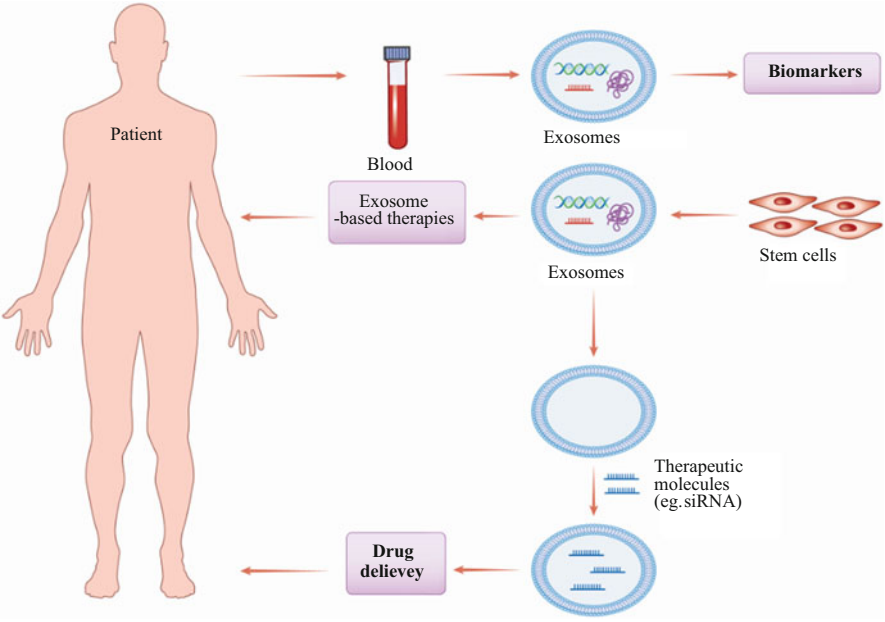

Fig. 2 Summary of the teranostic applications of exosomal miRNAs

of the most important characteristics of exosomes is that they can protect their payload due to lipid bilayered structure and their small size that allows them to remain in the circulation for a long time, thus allowing their detection over longtime periods (Syn et al. 2016). Once taken up by the recipient cells, the miR payload is successfully delivered by the exosomes to take part in the ongoing cellular processes (Barile and Vassalli 2017). Exosomes are also able to spread many pathogens, including viral proteins or viral genomes. The incorporation of pathogens into exosomes affects the immune response to infection (He et al. 2018). There are some limitations to exploit exosomes as a miR delivery system such as cellular toxicity due to extensive regulatory influence on various cellular processes and activities.

The current understanding of exosomal miRNAs has highlighted the importance of these microvesicles not only for cancer but also for other diseases. For example, different miR-21-5p, miR-29a-3p, and miR-126-3p are related to diabetic kidney disease process and progression that also tips them as prospective biomarkers of the disease (Assmann et al. 2018). miR-21 and miR-29a are also integral to the NSCLC growth and dissemination, while miR-126 fosters metastatic activity in hematological malignancies (Fabbri et al. 2012; Chevillet et al. 2014). However, the impact of exosomal miRNA release on tumor development remains to be determined. Exosomal miRs offer an effective pool of circulating miRs as compared to the non-exosomal miRNAs. Future studies are therefore required to discriminate the exosomal and free miRNAs besides determining whether miR exosomal packaging and uptake of miRNAs are specific and triggered by specific cues.

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Endothelial Progenitor Cells from Bench to Antitumor Therapy and Diagnostic Imaging

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Tiziana Annese, Roberto Tamma, and Domenico Ribatti

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Abstract

Cancer is the second leading cause of death worldwide after cardiovascular diseases, accounting for an estimated ten million deaths annually. Researchers are making a great effort to identify more efficient therapeutic strategies. To date, genetically modified stem cells are a potential candidate for the development of new antitumor therapies and diagnostic investigation methods.

Among stem cells, endothelial progenitor cells (EPCs), a subpopulation of multipotent hematopoietic stem cells (HSCs), appear promising. In response to specific stimuli, EPCs are fundamental to tumor progression because of their role in vasculogenesis and sprouting angiogenesis. In a healthy adult individual, the process of neoangiogenesis is activated only during wound healing and in the female uterus during ovulation. Therefore, it is reasonable to use them in anti-cancer therapy by taking advantage of their natural tropism to the altered micro-environment. Diverse studies demonstrated that EPCs predominantly home into the tumor mass, and hence, they are useful as a cellular vehicle for site-directed drug targeting to the tumors or for the delivery of imaging probe.

T. Annese · R. Tamma · D. Ribatti (✉)
Department of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy
e-mail: domenico.ribatti@uniba.it

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This chapter explores the underlying molecular mechanisms and the potential application of stem cell therapy in cancer with special reference to EPCs application in targeted gene therapy. How they could be modified, obtained in a significant amount, and administrated to treat cancer has been discussed.

Keywords

Angiogenesis · Cancer therapy · Diagnostic imaging · Endothelial · EPCs · Progenitor cells · Vasculogenesis

Abbreviations

5-FC	5-fluorouracil
Ac-LDL	acetylated low-density lipoprotein
ASCs	adult stem cells
BMMCs	bone marrow mononuclear cells
CD	cytosine deaminase
CEPCs	circulating EPCs
CFU-ECs	colony-forming unit-EC
DR4	death receptor 4
DR5	death receptor 5
ECFCs	endothelial colony-forming cells
ECs	endothelial cells
EC-SPs	endothelial cell-side progenitors
eNOS	nitric oxide synthase
EOCs	endothelial outgrowth cells
EPCs	endothelial progenitor cells
EPO	erythropoietin
ESCs	embryonic stem cells
FBS	fetal bovine serum
FSCs	fetal stem cells
GCV	ganciclovir
GMCSF	Granulocyte-macrophage colony-stimulating factor
GSCs	glioma stem-like cells
HIF-1α	hypoxia-inducible factor 1 alpha
HPCs	hematopoietic progenitor cells
HSCs	hematopoietic stem cells
iPSCs	induced pluripotent stem cells
MAPCs	multipotent adult progenitor cells
MCP-1	monocyte chemotactic protein
MRI	magnetic resonance imaging
MVD	microvessel density
NK	natural kill cells
PARP	poly ADP-ribose polymerase
PBMCs	peripheral blood mononuclear cells

PD-1	programmed death-1
PEI2k	polyethylenimine 2 kDa
PlGF	placental growth factor
PSCs	placenta stem cells
QQc	quality and quantity culture
SCs	stem cells
SDF	stromal cell-derived factor
Sirt1	sirtuin-1
SPECT	single-photon emission computed tomography
SPIO	superparamagnetic iron oxide
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
UEA1	Ulex europaeus agglutinin 1
uPAR	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VESCs	vascular endothelial stem cells
VW-EPCs	vascular wall endothelial progenitor cells
vWF	von Willebrand factor
VW-VSCs	vascular wall-resident vascular stem cells

Introduction

Cancer is a vast group of diseases that share some characteristics. Cancer cells can develop in all tissues/organs of the body, have a high proliferation rate, and can invade the normal surrounding tissue and beyond. Metastasizing is a leading cause of death from cancer (Dillekas et al. 2019). Cancer is the second dominant death source worldwide after cardiovascular diseases, accounting for an estimated ten million deaths annually (Bray et al. 2018). Lung, breast, colorectal, prostate, stomach, liver, esophagus, cervix uteri, thyroid, and bladder cancers are, in order, those with the highest incidence (Bray et al. 2018). As a general trend, patients' survival rate and life quality are improving thanks to early diagnosis, prevention campaigns, and improved standards of care. Despite this, patients' physical and economic efforts and the entire health system make cancer a huge problem and a considerable challenge for researchers.

Different therapeutic designs are distinguished according to the type of cancer and the stage of development. These include surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant, precision, and personalized medicine (NCI 2020). Surgery for cancer treatment is called curative surgery and is usually applied when the tumor mass is well-confined to a specific body part. Before and/or after resection, the patient could be treated with radiotherapy or chemotherapy. Radiation therapy includes different approaches such as external beam radiation therapy, internal radiation therapy (brachytherapy), oral or systemic radiation therapy, and photodynamic therapy. The operating principle consists of high-energy electromagnetic waves or molecules that create DNA

damage in proliferating tumor cells. Chemotherapy consists of using one or more drugs that act mainly against proliferating cells and thus against cancer cells to prevent or limit their growth and spread. Immunotherapy is a treatment that uses cells or molecules of the immune system, such as use of antibodies or vaccines, or T-lymphocytes, to restore or boost the patient's immune system. Targeted therapy applies drugs designed to "target" specifically cancer cells or cells of the surrounding microenvironment without affecting normal cells, exploiting their unique expression of some genes or proteins. Hormone therapy is a systemic one in which hormones are administrated to destroy cancer that depends on them to grow, like breast and prostate cancers that depend on sex hormones. Stem cell transplant is exploited to replace the patient's bone marrow cells treated with chemo and/or radiation therapy against such cancers as leukemia and lymphoma. Precision and personalized medicine is the newest approach and is based on the patient's genome and epigenome characterization because there is high intra-tumoral heterogeneity. Still, it is only in clinical trials for now.

Among these approaches for cancer treatment and to adopt high-performance methods in terms of improved therapeutic efficacy and fewer undesirable effects, stem cell transplant, alone or in combination with other therapies, could be the right strategy for treatment and the development of new diagnostic investigation methods due to its enhanced target on tumors.

Stem Cells

In all development stages from the embryo to the adult, all organs and tissues possess undifferentiated precursor cells, mitotically active, multipotent, and capable of regenerating mature cells, called stem cells (SCs). They are a reservoir of precursor cells playing a homeostatic role essential for replacing dead or damaged cells due to trauma or diseases (Galli et al. 2003). SCs are highly undifferentiated cells that do not possess morphological, structural, molecular, and antigenic characteristics found in the tissue's differentiated cells to which they belong.

SCs can perpetuate themselves through their ability to self-renew (Weissman et al. 2001). In general, the in vivo self-renewal last for the organism's whole life, but in vitro, it is unlimited under the appropriate experimental conditions. Two types of stem cell divisions are distinguished, symmetric cell divisions and asymmetric ones (Shahriyari and Komarova 2013). In symmetric divisions, the two daughter cells are identical to each other and to the mother cell (expansive symmetric division) or, in the alternative, identical to each other but different from the mother (differentiative symmetric division), called progenitors. In asymmetric division, a stem cell produces one differentiated cell and one stem cell. This system allows the number of stem cells to remain constant at the end of each cell generation. It offers the enormous advantage of increasing or decreasing the number of stem cells within a tissue.

Another critical feature of stem cells is multipotentiality, which is the ability to give rise to a differentiated progeny comprising all types of cells of the residence tissue or, in the case of embryos, to all cells of the adult organism. Stem cells,

according to their potential, are classified as totipotent if they are not specialized and can give rise to a new embryo, such as embryonic cells at the stage of 4–8 cells after 4–5 days from fertilization; pluripotent, if they have the potential to differentiate into all cell types that derive from the three embryonic layers (endoderm, ectoderm, and mesoderm), but they do not have the potential to give rise to an embryo, such as embryonic stem cells (ESCs) at the blastocyst stage with 20–30 cells, after 5–7 days from fertilization; multipotent, if they can differentiate in all cell types of a specific organ or tissue such as hematopoietic stem cells (HSCs); unipotent, if they can give rise to a single cell type such as keratinocytes (Łos et al. 2019).

SCs can also be recruited where they are required to participate in the repair process, thanks to a controlled process called homing, and once they reach the site, they settle there (engraftment). Their well-directed migration is under the control of cytokines gradient and is used to regenerate damaged tissues.

Based on the source, stem cells are classified into ESCs, such as cells isolated from human blastocysts; fetal stem cells (FSCs), such as gonadal cells from abortive fetuses; umbilical cord stem cells, such as cells isolated from cord blood umbilical of newborns; placenta-derived stem cells (PSCs), isolated from the placenta of newborns; adult stem cells (ASCs), isolated from adult tissues such as HSCs; induced pluripotent stem cells (iPSCs), obtained by dedifferentiation of mature cells to embryonic cells by genetic manipulation. iPSCs open new therapeutic opportunities, which are practically the same as those of human embryonic stem cells, but without ethical and scientific concerns.

ASCs, present in small quantities in stem niches of the whole organism, remain quiescent until disease or trauma reactivate them inducing proliferation and differentiation. The niche is a tissue location where a dynamic and specialized microenvironment regulates stem cell biology (proliferation, maintenance, or differentiation). They are present in different organs and tissues: the hematopoietic system (Osawa et al. 1996), brain (Galli et al. 2000; Goritz and Frisen 2012), dermis (Toma et al. 2001), muscle (Qu-Petersen et al. 2002), and liver (Shafritz et al. 2006). Until recently, it was generally thought that ASCs could at most differentiate into all cell types of the tissue they belong to (Price et al. 2007). However, today, it has been observed that, under optimal set of conditions, they can differentiate into other cell types, in addition to those of the original tissue. For example, after bone marrow transplantation enriched with HSCs, they can differentiate in all the three germinal layers' cells (Jackson et al. 2001; Mezey et al. 2000; Orlic et al. 2001; Theise et al. 2000).

Stem cells are applied in regenerative medicine for diseases such as Parkinson's disease (Ourednik et al. 2002), spinal cord damage (Teng et al. 2002), multiple sclerosis (Pluchino et al. 2003), amyotrophic lateral sclerosis (Clement et al. 2003), stroke (Liu et al. 2009), retinal degeneration (Li et al. 2006), Alzheimer's disease (Barnham et al. 2004), myocardial infarction (Jackson et al. 2001), and others. The unique self-renewal and differentiation potential of stem cells are the primary reasons for their use to regenerate damaged organs and correct congenital diseases. However, a major limitation for the therapeutic use of stem cells is the risk of iatrogenic oncogenesis.

The source of the cells for therapy could be the same patients (autologous transplantation) or a donor (allogeneic transplantation). The main attraction is for the immune-privileged autologous stem cells that express the major histocompatibility complex 1 (MHC1), but not MHC2, clusters of differentiation because these can be used in immunocompetent patients, avoiding side effects and with better therapeutic efficacy and significantly improved safety. For instance, in a preclinical study to evaluate EPCs for target gene therapy, it was shown that these cells do not express MHC-I, are resistant to lysis by non-activated natural kill cells (NK), and survive and participate in tumor blood vessel formation after intravenous injection (Wei et al. 2004).

An Overview of Endothelial Progenitor Cells

EPCs are mostly unipotent stem cells capable of differentiating into endothelial cells (Khakoo and Finkel 2005). In vivo, they can differentiate from hemangioblasts, bone marrow multipotent adult progenitor cells (MAPCs), and myeloid/monocytic cells. In vitro, the early and late EPCs are distinguished (reviewed in (George et al. 2011)). Furthermore, to be present as such in bone marrow, peripheral and umbilical cord blood, EPCs can be produced by transdifferentiation of stem cells present in various tissues and organs, under the influence of adequate microenvironments for endothelial differentiation (for extensive EPCs sources, readers can consider the reviewing article (Chopra et al. 2018)).

EPCs express endothelial markers such as CD133, CD31, CD34, CD146, and VEGFR2 and do not express the hematopoietic marker CD45 or mature ECs markers including VEGFR1, VE-cadherin, and Von Willebrand factor (vWF) (George et al. 2011; Medina et al. 2017). $CD34^+/CD133^+/VEGFR2^+$ cells are usually, but not unambiguously considered EPCs (Medina et al. 2017).

EPCs can be studied in two ways, flow cytometry or in vitro culture (Medina et al. 2012). Flow cytometry is used for studying circulating EPCs (CEPCs) in the blood samples where they are quantified as the percentage of mononuclear cells $CD34^+/VEGFR2^+/CD133^+$ (Peichev et al. 2000; Wu et al. 2007). In vitro culture methods are applied to study EPCs derived from peripheral blood mononuclear cells (PBMCs) or by direct flushing of bone marrow mononuclear cells (BMMCs) and expanded using endothelial-specific media. During in vitro culture, two different cell types can be generated, the early and late outgrowth cells being hematopoietic and endothelial, respectively (see Table 1) (Medina et al. 2010). Only the last ones are considered valid EPCs (see Table 2) (Banno and Yoder 2019). The late outgrowth cells, also called endothelial colony-forming cells (ECFCs), originate from $CD45^-/CD133^-/CD34^+$ MNCs, in vitro arise after 7 days, have a highly proliferative polygonal shape, do not differentiate into hematopoietic cells, and produce vascular tube in vitro and in vivo. Moreover, ECFCs can affect neovascularization in vivo, take up acetylated LDL, bind to Ulex europaeus agglutinin 1 (UEA1), and express the surface markers CD31, vWF, CD105, CD146, VE-cadherin, and VEGFR2 (Timmermans et al. 2009; Yoder et al. 2007). Some studies have shown a synergistic

Table 1 EPCs isolated using *in vitro* culture methodologies classification based on a specific phenotype and a biological function

	Name	
In vitro	MACs; early outgrowth EPCs; early EPCs; hematopoietic EPCs; CACs; PACs; CFU-ECs; CFU-HILL; small EPCs; Myeloid EPCs	ECFCs; late outgrowth EPCs; LATE EPCs; non-hematopoietic EPCs; OECs; BOECs' EOCs; large EPCs
From PBMCs or umbilical cord blood appear after	4–10 days	>2 weeks
Achieve peak growth at	2–3 weeks	4–8 weeks
Survive up to	4 weeks	12 weeks
Markers	$CD45^+$ $CD14^+$ $CD31^+$; $CD146^-$ $CD34^-$	$CD31^+$ $CD105^+$ $CD146^+$ VE-$cadherin^+$ vWF^+ $VEGFR2^+$; $CD45^-$ $CD14^-$
Role in new vessel formation	Do not differentiate into ECs but promote angiogenesis through paracrine factors that indirectly augmented proliferation, migration, and the tube forming capability of ECFCs	Became ECs and participate in new blood vessel formation or vascular repair
Secretion/ expression of pro-angiogenic factors	VEGF; IL-8; MMP9	VEGFR2; CXCR-1; MMP2

The table shows the complex EPCs nomenclature and the two leading EPCs population features studied for their pro-angiogenic properties

Abbreviations: *BOECs* blood outgrowth ECs, *CACs* circulating angiogenic cells, *CFU-ECs* colony-forming unit-EC, *CFU-HILL* colony-forming unit HILL EPC, *ECFCs* endothelial colony-forming cells, *EOCs* endothelial outgrowth cells, *MACs* myeloid angiogenic cells, *PACs* pro-angiogenic hematopoietic cells, *PBMCs* peripheral blood mononuclear cells, *OECs* outgrowth ECs

effect of early and late outgrowth cells when used together compared with one of the two cells alone as cell therapy (Pearson 2010; Yoon et al. 2005).

Both early and late EPCs promote angiogenesis. Early EPCs contribute to new vessels formation by secreting a series of growth factors and cytokines, such as VEGF, stromal cell-derived factor-1 (SDF-1), granulocyte colony-stimulating factor (G-CSF), and insulin-like growth factor 1 (IGF-1), which stimulate ECs proliferation and survival, and direct endogenous progenitor cell recruitment into sites of neo-vascularization (Urbich et al. 2005). Furthermore, early-EPCs provide relevant protective effects to themselves and differentiated EPCs from apoptosis under oxidative conditions in an auto- or paracrine manner, recruiting other cells within the peripheral blood (Yang et al. 2010). Late EPCs directly contribute to vasculogenesis by providing structural support via differentiation into mature ECs (Hur et al. 2004). They can also promote angiogenesis by the secretion of numerous cytokines (Moubarik et al. 2011).

Table 2 How to distinguish "bona fide" EPCs?

	MACs	ECFCs	ECs
Potency Assys (capacity to form a vascular network in vitro and in vivo)	Only in conditioned media	Intrinsic tube forming capacity	Intrinsic tube forming capacity
Detailed identity immunophenotype	$CD14^+$	$CD31^+$	$CD31^+$
	$CD31^+$	$CD34^+$	VE-$cadherin^+$
	$CD45^+$	$CD105^+$	$VEGFR1^+$
	$CD34^-$	$CD133^+$	vWF^+
	$CD146^-$	$CD146^+$	$CD34^-$
		VE-$cadherin^+$	$CD133^-$
		$VEGFR2^+$	
		vWF^+	
		$CD14^-$	
		$CD45^-$	
Clonogenicity capacity: (single-cell colony-forming)	Lack	High	Lack
Proliferative capacity	Medium	High	Low

The table shows the tests that must be performed to identify bona fide *EPCs unequivocally. The term EPCs should be restricted to only those cells that display vessel-forming potential, the right immunophenotype, and have high clonogenic potential and proliferation rate*
Abbreviations: *ECFCs* endothelial colony-forming cells, *ECs* endothelial cells, *MACs* myeloid angiogenic cells

Under physiological conditions, EPCs homing-in is aimed to maintain vascular integrity during repair of damaged tissues, restore organ function, and participate in postnatal angiogenesis (Asahara et al. 1997, 1999a; Urbich and Dimmeler 2004). However, EPCS' vasculogenic potential is also exploited by tumors to facilitate their progression (Asahara et al. 1999a; Dong and Ha 2010). As shown in preclinical research, in response to endogenous and exogenous signals, $VEGFR2^+$ EPCs can get mobilized from the bone marrow into the peripheral blood circulation and subsequently home-in to tumor neovascularization sites where they differentiate into ECs, thus contributing to angiogenesis (Nolan et al. 2007; Rafii et al. 2002). Endogenous signals released from tumor cells and their microenvironment induce hypoxia-inducible factor 1-alpha (HIF1-α) overexpression, glucose reduction, and reactive oxygen species increase. These events promote the release of VEGF, SDF-1, monocyte chemotactic protein (MCP-1), and erythropoietin (EPO), which facilitate EPCs homing-in to neovascularization sites (Annese et al. 2019; Dong and Ha 2010; Ribatti 2004). More precisely, this occurs before the angiogenic switch in the avascular tumor phase (Gao et al. 2008). Once recruited, the EPCs can directly participate in new blood vessel formation or can merely release pro-angiogenic factors. The neoangiogenesis is also sustained by co-mobilization of $VEGFR1^+$ hematopoietic progenitor cells (HPCs), which home-in to the tumor-specific pre-metastatic sites and form cellular clusters, the so-called pre-metastatic niche (Kaplan et al. 2005). There is convincing evidence from both preclinical and clinical studies that exogenous signals, such as disruptive vascular agents, chemotherapy,

and surgery, might induce an acute release of EPCs from bone marrow, contributing to tumor growth (Bertolini et al. 2003; Furstenberger et al. 2006; Roodhart et al. 2009; Shaked et al. 2008). Of particular importance is the ability of EPCs to home-in not only into the tumor's vasculature but also into the tumor proper.

Endothelial Progenitor Cells in Neovascularization

The development of EPCs-based therapies to induce or suppress new blood vessel formation necessitates the comprehension of cellular and molecular mechanisms of neovascularization. EPCs have a role in both vasculogenesis and angiogenesis. Physiological vasculogenesis is also known as developmental vasculogenesis because it occurs during embryo development. From hemangioblast, which is a common precursor of hematopoietic and vascular systems, EPCs differentiate in the bone marrow and then extravasate into the peripheral circulation in response to VEGF/VEGFR2 stimuli. From circulation, EPCs follow the stimuli gradient and upon arrival at the site of injury, they differentiate into mature ECs and participate in the ongoing vascular development (Masuda and Asahara 2003). In the adult, these EPCs from the bone marrow could participate in physiological blood vessel formation and pathological one during the early phase of tumor neovascularization.

Vasculogenesis involves the recruitment and participation of circulating cells, and de novo formation of blood vessels from these cells, while angiogenesis results from the proliferation of existing blood vessels. To be more precise, two types of angiogenesis are distinguished as sprouting and non-sprouting angiogenesis. Sprouting angiogenesis occurs when ECs migrate (tip cells) toward the VEGF gradient source and proliferate (stalk cells) to form abluminal sprouts that subsequently fuse and generate new vessels (Risau 1997; Uccelli et al. 2019). On the other hand, non-sprouting angiogenesis, or intussusceptive angiogenesis, occurs in the absence of a gradient, and all ECs respond to VEGF by assuming a stalk phenotype. During intussusception, an already existing vessel splits into two by forming intraluminal endothelial pillars, which fuse longitudinally (Risau 1997; Uccelli et al. 2019). Angiogenesis plays an essential role throughout embryonic development, besides wound healing, tissue ischemia, and tumor vasculature formation during postnatal life. Hence, it is now being exploited as a novel therapeutic target in cancer treatment. During angiogenesis, EPCs can indirectly contribute to tumor vascularization via autocrine/paracrine mechanisms (Asahara et al. 2011).

In addition to the extravasation of EPCs from the bone marrow and homing-in to the site of injury, the neovascularization process is also supported by immature cells present in the vascular wall of various organs. These cells are called vascular wall-resident vascular stem cells (VW-VSCs) that differentiate in smooth muscle cells and ECs (Tamma et al. 2020; Torsney and Xu 2011). The subpopulation called vascular wall EPCs (VW-EPCs) differentiate in ECs and are also known as endothelial cell-side progenitors (EC-SPs) $CD200^{+}/CD157^{+}$ (Ingram et al. 2005; Wabik and Jones 2015). In hypoxia conditions, these cells are under self-renewal and differentiation to stalk cells contributing to long-term ECs proliferation and, thus, angiogenesis (Takakura 2018).

Given the ubiquitous EPCs' role in neovascularization, their concentration in peripheral blood can be a surrogate biomarker indicating vasculogenic/angiogenic tumor activity and therapy efficacy on tumor vasculature as currently done with microvessel density (MVD) and VEGF expression (Bianconi et al. 2020; Nico et al. 2008; Schluter et al. 2018). EPCs concentration is advantageous because it is accurately but noninvasively compared to MVD and VEGF evaluation, but it is disadvantageous because only 0.025% of the PBMCs are EPCs (Peichev et al. 2000). The small amount limits the translation of prosperous findings of EPCs from bench to practical use. EPCs amount is even less if EPCs from VESCs in the preexisting blood vessels are considered. Therefore, for EPCs-based therapy, they should be first expanded ex vivo.

Endothelial Progenitor Cells Applications

EPCs contribute to tissue regeneration processes via neovascularization through paracrine mechanisms or differentiation in mature ECs (Asahara et al. 1999a; Kalka et al. 2000). The freshly isolated autologous PBMCs or BMMCs have been applied to clinical vascular regenerative therapy in patients with peripheral arterial disease, critical limb ischemia, or myocardial infarction (Deutsch et al. 2020; Koshikawa et al. 2006; Kudo et al. 2003; Lara-Hernandez et al. 2010; Li et al. 2016a; Liotta et al. 2018). These researches indicated that cell-based therapy was safe, feasible, and useful.

Potential stem cell applications against cancer have been well-reviewed elsewhere (Chu et al. 2020) are for cell transplantation, post-cancer treatment, vaccine production, therapeutic carriers, or immune cells generator. Clinically, EPCs can be employed in three different manners: (i) for neovascularization; (ii) as target cells in anti-EPCs therapy against tumors; (iii) as biomarkers for disease identification and severity (Chopra et al. 2018).

With the purpose of neovascularization, vascular EPCs can be exploited for their ability to release several angiocrine growth factors, or other bioactive molecules, to maintain and sustain tissues/organs' regeneration, for example, by increasing the releasing of oxygen and nutrients through neoangiogenesis. EPCs present in preexisting blood vessels or recruited from bone marrow could be used in vascular regeneration therapy in many diseases like revascularization of ischemic tissues after heart infarction (Huang et al. 2013; Moubarik et al. 2011; Steinle et al. 2018). EPCs could be applied to non-angiogenic and angiogenic tumors to induce blood vessel formation, which will be a direct access route of the drug on the tumor cells, or induce blood vessel normalization, which will alleviate hypoxia and pro-tumor microenvironment, respectively (Collet et al. 2016).

As potential therapeutic carriers, a Trojan horse, EPCs combined with targeted antiangiogenic drugs for cancer treatment act as a delivery vehicle that protects the therapeutic agents from rapid biological degradation, reduce systemic side effects, and increase local therapeutic levels due to the intrinsic tumor-targeting effect. Recent advances in cellular engineering have led to stem cell-based vector development to serve as a vehicle for angiogenesis inhibitors or genes directly into the tumor

endothelium (Janic and Arbab 2010; Nakamura et al. 2004). These novel approaches are useful in oncology to selectively destroy cancer cells, leaving healthy cells unaffected thus alleviating the side effect of cancer therapy (Chong et al. 2016; Ruggeri et al. 2018). For instance, in Sprague-Dawley rats, EPCs isolated from PBMCs were genetically modified to induce a stable expression of antiangiogenic endostatin, reducing VEGF expression. These genetically modified EPCs were successfully tested to suppress retinal vascular leakage and could be advanced for clinical assessment because endostatin overexpression may serve as a potential therapeutic agent (Ai et al. 2018).

Antitumor treatments' efficacy is usually evaluated by imaging techniques such as X-ray, computed tomography, magnetic resonance imaging (MRI), and ultrasound. EPCs can be used for diagnosis, prognostic prediction, and follow-up. EPCs can be labeled for CD133 for tracking their in vivo fate after injection by MRI for diagnosis and follow-up. As biomarkers of tumor development and/or progression, several studies have demonstrated clinical correlations between CEPCs concentration and tumor stage (Nowak et al. 2010; Ramcharan et al. 2013; Yu et al. 2013), tumor size (Richter-Ehrenstein et al. 2007; Su et al. 2010), VEGF serum concentration (Rafat et al. 2010; Yang et al. 2012), and MVD (Li et al. 2018a; Maeda et al. 2012). During hematological malignancies' comparison with solid tumors, many studies have demonstrated a close association between EPCs and disease activity, so much so that circulating EPCs are useful diagnostic, therapeutic, and prognostic biomarker (Ge et al. 2015; Ruggeri et al. 2018).

The EPC-based therapies are much better known for cardiovascular diseases compared to oncological ones. EPCs were proposed to induce angiogenesis in ischemia (Li et al. 2018b; Zheng et al. 2014), for post-injury vascular endothelial regeneration (Abd El Aziz et al. 2015; Guo et al. 2017), and ex vivo tissue engineering (Sales et al. 2010). However, the enthusiasm for the possible applications of EPCs in clinical therapy is severely limited by the lack of in-depth characterization and understanding of early and late outgrowth EPCs.

Endothelial Progenitor Cells Sources, Ex Vivo Culturing, and Implantation

Mouse, monkey or human ESCs, fetal liver, human umbilical cord blood, bone marrow, and peripheral blood might be used as the potential EPCs sources (Debatin et al. 2008; Zakrzewski et al. 2019). The use of stem/progenitor cells from embryos is advantageous and ideal because they can show unlimited and undifferentiated proliferation and evade immunological rejection as they do not express MHC-I. Still, there are ethical considerations and risk of malignant transformation that restrict their progress to the clinical setting (Wei et al. 2004; Werbowetski-Ogilvie et al. 2009). EPCs derived from the fetal liver can be easily isolated and cultured; however, the clinical applicability of these cells is limited by the challenges of creating fetal liver tissue banks and host immune incompatibility (Cherqui et al. 2006).

Stem/progenitor cells present in umbilical cord blood have a higher proliferative capacity, readily available, and easy to isolate than adult bone marrow-derived cells.

However, umbilical cord donation has yet to achieve widespread acceptance, besides the chance of immunologic graft-versus-host disease in the recipients (Ingram et al. 2005; Murohara 2001; Murohara et al. 2000; Qin et al. 2017).

Also, multipotent adult progenitor cells isolated from postnatal bone marrow have extensive proliferation potential ex vivo and can differentiate into mesodermal lineage cells as EPCs (Reyes et al. 2002). Thus, they can be effectively applied in autologous therapy, thanks to potentially low-level immune recognition and destruction of these cells by the host immune system.

More recently, EPCs have been isolated from human adult somatic cells, that is, fibroblasts, through transdifferentiation into iPSCs (Purwanti et al. 2014; Taura et al. 2009). Nevertheless, like ESCs, EPCs iPSCs-derived will need an in-depth characterization to exclude tumorigenic potential. An easily accessible EPCs' source is either peripheral blood or bone marrow. Circulating autologous EPCs could be isolated from these sources using markers like CD34, CD133, or VEGFR2 (Asahara et al. 1997; Shi et al. 1998). Circulating EPCs and bone marrow-derived EPCs are among the least complicated sources to use, but the main obstacle in their use in regenerative medicine is low quality and quantity, and immune recognition (Asahara et al. 2011; Sukmawati and Tanaka 2015). Chemotactic molecules as VEGF, placental growth factor (PlGF), granulocyte-macrophage colony-stimulating factor (GMCSF), or statins are used to treat patients/donors to increase EPCs number (Asahara et al. 1999b; Dimmeler et al. 2001) (for schematic EPCs application as therapy see Fig. 1).

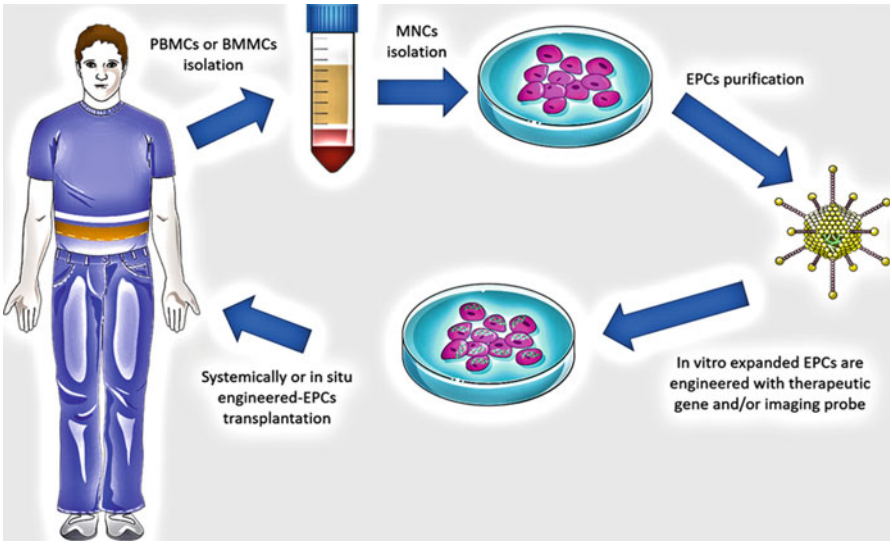

Fig. 1 Schematic representation of EPCs as cellular vehicles. EPCs derived from PBMCs or BMMCs are expanded and transduced *in vitro* to express pro-drugs or tratted for the expression of imaging probe can be systematically or in-situ replanted in the patients.

EPCs, after isolation from PBMCs or BMMCs, are expanded in vitro. There are several optimized protocols available for ex vivo culture and expansion of EPCs. However, all of these protocols require that EPCs are plated on extracellular matrix proteins coated dishes and maintained in endothelial basal medium with added supplements and growth factors (Au et al. 2008; Kawamoto et al. 2001; Lu et al. 2014). Concerning supplements, EPCs' expansion is expensive and time-intensive due to high concentrations and frequent supplementation of growth factors because of their short half-life at physiological temperatures (Khalil et al. 2020). Moreover, supplements, such as fetal bovine serum (FBS), could be unsafe for clinical application due to their animal origin, prone to batch-to-batch variations, xenoimmunization, and possible contamination of mycoplasma, viruses, endotoxins, and prions (Dessels et al. 2016). The development of optimal protocols to expand EPCs without growth factors is a promising approach to simplifying clinical translation. Interestingly, polyphenols benefit EPCs number and functional activity (Di Pietro et al. 2020; Huang et al. 2010). For example, an attractive agent to expand EPCs is the natural flavonoid quercetin. It increases the number and functional activity of EPCs and protects them against serious glucose-induced damage by inducing Sirtuin-1 (Sirt1)-dependent endothelial nitric oxide synthase (eNOS) upregulation (Zhao et al. 2014). To avoid the use of animal serum, several laboratories have developed novel serum-free expansion methods enriched with optimal cytokine and growth factor combinations (Hagiwara et al. 2018; Kado et al. 2018; Masuda et al. 2012). These methods are known as the quality and quantity culture (QQc) system and ensures optimizing EPCs-based therapy by augmenting their qualitative and quantitative vasculogenic properties and providing measurable regenerative capacity (Sukmawati and Tanaka 2015).

To isolate and expand EPCs, it is imperative to consider that EPCs are decreased in number and functional activity related to age and cardiovascular risk factors (Huang et al. 2014; Kaur et al. 2018). Therefore, isolation and application of EPCs from patients with these backgrounds have a high chance of receiving EPCs with low therapeutic effect.

Before administration, expanded EPCs are characterized for their morphology, surface markers expression by flow cytometric analyses, eNOS levels, and Ac-LDL uptake/fluorescein isothiocyanate (FITC)-lectin binding actives. Moreover, their routine functional characterization also involves assessment of angiogenic potential by in vitro tube formation assay or in vivo by chick chorioallantoic membrane (CAM) assay (Kukumberg et al. 2020; Merckx et al. 2020; Qin et al. 2018; Song et al. 2010). Furthermore, recent studies advise performing isolated cells' efficacy and safety, for example, by transplantation in a nonhuman primate model (Qin et al. 2018).

Once collected sufficient number in vitro, EPCs can be conditioned to enhance their functionality for more efficient functionality and therapeutic benefits. Several studies about the revascularization of ischemic tissues have employed various growth factors or recombinant proteins or genes using nano- or microparticles to improve tissues' revascularization and upregulate pro-angiogenic proteins (Bhise et al. 2011; Simon-Yarza et al. 2012). Recombinant proteins are costly, and it is not

easy to maintain adequate protein levels at the target region due to their relatively short half-lives (Gupta et al. 2009). Gene therapy with viral and nonviral delivery system was applied as an alternative strategy to express the desired pro-angiogenic proteins, and it has shown to be promising.

EPCs can be genetically manipulated by stable transduction using retrovirus or lentivirus-based vectors encoding for the gene/s of interest, allowing a long-term transgene expression. Besides their high transduction efficiency, their use is convenient when EPCs are used as a vehicle due to their susceptibility to gene transduction protocols. However, since viral vectors are usually replication-defective, there will be a physiological clearance reduction of EPCs vehicle following administration. This reduction can be advantageous if a temporary rather than stable presence of the cells is the goal of cell therapy. EPCs can also be modified with non-integrating viral vectors such as adenovirus or herpes simplex virus, or plasmid vector, inducing short-term effects. Moreover, using a non-integrating method, the EPCs can be modified with synthetic mRNAs, which can express exogenous proteins without the hazard of insertional mutagenesis since the delivered mRNAs remain in the cytoplasm for translation without passing into the nucleus (Sahin et al. 2014; Steinle et al. 2018). The synthetic mRNA transfection leads to the transient production of exogenous proteins of interest in the cells, and subsequently undergoes natural degradation thus leaving no traces of the delivered mRNA.

After isolation, modification, and characterization, EPCs can be implanted in patients. Generally, stem cells could be introduced in different ways, such as intravenous, intramuscular, intra-articular, and intrathecal (Saeedi et al. 2019). Intravenous is the safest and most straightforward method to deliver the EPCs throughout the body. This way is a preferred route administration as it is simple and feasible, does not require general anesthesia, and allows administration of repeated doses at different times (Haider et al. 2017). The transplanted EPCs mainly home-in to a tumor site due to attraction to the tumor vasculature by its angiogenic drive, but the efficiency is not 100%. In effect, most cells intravenously inject end up in the non-target sites, including lungs, liver, and spleen (Leibacher and Henschler 2016; Varma et al. 2013b). Several factors could explain the lack of efficiency, including tumor microenvironment composition variability, vascular network size, or angiogenic stimulus power (Li et al. 2011; Wang et al. 2016). Moreover, after intravenous administration, EPCs have to compete with the endogenous EPCs for incorporation into the target organ such as a tumor. This necessitates the suppression of endogenous EPCs. One of the strategies for improving EPCs tumor homing is to deliver these cells as an adjuvant to chemotherapy or radiation therapy because this can increase the migration and incorporation of EPCs into the tumor (Shaked et al. 2008). Furthermore, to improve EPCs delivery and homing-in capacity, they might be directly injected into the arterial circulation or infused at multiple time-points (Dudek 2010; Lin et al. 2020).

To successfully translate EPCs into cell-based therapy for routine patient application, it is critical to develop a technique to monitor transplanted cells' in vivo biodistribution after delivery. Among the in vivo cell-tracking and cell-fate determining techniques, magnetic resonance imaging (MRI) is one of the most powerful

one because of its satisfactory resolution (Aicher et al. 2003; Hu et al. 2012; Wang et al. 2003). For MRI, the cells are labeled with MRI contrast agents that are not efficiently loaded into cells to avoid cytotoxicity (Crabbe et al. 2010; Hu et al. 2012). An alternative label is the paramagnetic agent IronQ, a complex of iron and quercetin, added to cell culture (Kantapan et al. 2017). It is not only traceable by MRI but also serve as cell proliferation inducer (Kantapan et al. 2017).

Endothelial Progenitor Cells in Preclinical Cancer Studies

Searching for clinical trials involving EPCs in cancer therapy, it is possible to find only five studies, all providing information regarding the characterization and quantification of CEPCs using biomarkers (ClinicalTrials.gov Identifier: NCT00393341; NCT00753610; NCT00325871; NCT00826683; NCT00067067). These studies are based on complete evidence about the emerging role of CEPCs in tumor angiogenesis as surrogate markers of antiangiogenic therapies efficacy.

It is widely known that neovascularization is a crucial cancer hallmark that facilitates cancer cells proliferation and progression. Blood vessels deliver oxygen and nutrients to cancer cells allowing them to grow further 2 mm in diameter. When tumor mass is over, in response to hypoxia and microenvironment signals, cancer cells overexpress molecules that promote vasculogenesis, angiogenesis, and evasion. As discussed earlier, resident ECs and EPCs, which line all blood vessels or are present in the peripheral circulation respectively, or are recruited from bone marrow migrate toward an angiogenic cue, proliferate and form new vessels. EPCs' involvement in tumor vasculogenesis, contributing to the development of vascular network or vascular mural cells, and their homing-in to the site of tumor angiogenesis means that they have access to distant and, in most cases, undetectable micrometastases are the reasons behind the use of these cells in cancer therapy (Rajantie et al. 2004; Reyes et al. 2002).

In an experimental mouse orthotopic hepatoma model developed using tumor liver cell line HepG2, Zhu et al. (2012) demonstrated that intravenous tail-vein injection of BMMCs-derived EPCs preferentially migrated into the site of tumor development (liver) compared to the other organs. They also demonstrated that EPCs migrated to the tumor site in response to the cytokines (VEGFR, HIF-1α, SDF1) cues secreted by the tumor cells. On the contrary, some researchers have reported failure of EPCs' chemotaxis in all kinds of tumors in response to the chemical cues emanating from the tumor cells (Annabi et al. 2004; De Palma et al. 2005; Larrivee et al. 2005; Lyden et al. 2001; Purhonen et al. 2008).

The orientated homing of EPCs in hepatomas as in other tumors enhances their possible clinical applications as delivery vehicles for suicide gene therapy, anti-angiogenesis gene therapy, or tumor suppressor gene therapy. An effective EPCs-based strategy in cancer therapy is to genetically manipulate EPCs with the genes encoding for the enzymes that metabolize pro-drugs into pharmacologically active anticancer drug derivatives that would kill the surrounding cancer cells based on a spectator effect (bystander effect) (Freeman et al. 1993; Zweiri and Christmas 2020).

The death of the donor EPCs and their ineffectiveness against rapidly growing or large tumor limit these suicide gene therapies after drug activation (Wei et al. 2007). During the last few years, the effectiveness of an exciting tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) anticancer activity has been reported (Lim et al. 2015; Yuan et al. 2018). TRAIL initiates the pro-apoptotic pathway by selectively binding with its death receptors-4 and -5 (DR4, DR5), while sparing the healthy cells unaffected (Forster et al. 2013; Kichev et al. 2014). Deng et al. (2018) engineered EPCs (isolated from PBMCs of neonatal Sprague-Dawley rats) with a lentivirus encoding for TRAIL for glioma treatment. TRAIL has a short half-life and also fail to cross through the blood-brain barrier and (Guo et al. 2011; Holoch and Griffith 2009). Thus, EPCs-based TRAIL gene delivery has overcome these problems (Choi et al. 2016; Redjal et al. 2015; Wang et al. 2014). TRAIL-EPCs migrate to glioma cells SHG44 in the transwell assay and induce glioma cell apoptosis in a co-culture in vitro system by increasing the cleaved caspase-3 and -8 levels and poly ADP-ribose polymerase (PARP) (Deng et al. 2018). To solve EPCs ineffectiveness due to their small number, an indirect strategy is to target tumor vasculature cells, which are critical for tumor growth and survival, instead of the whole enormous tumor cell mass. For instance, Dudek et al. (2007) have shown that genetically engineered EPCs over-expressing the antiangiogenic molecules endostatin significantly decreased tumor vascularization and growth after tail vein injection into NOD-SCID vein mice with subcutaneously implanted Lewis lung carcinoma cells. Laurenzana et al. (2014) developed a personalized therapy against melanoma using autologous MMP12-engineered EPCs to treat both tumor cells and tumor vasculature.

MMP12 is a metalloelastase with a bivalent role: protective if expressed by macrophages and non-protective if expressed by tumor cells (Houghton et al. 2006; Margheri et al. 2011; Martin and Matrisian 2007). MMP12 application as an anticancer strategy is based on MMP12's enzyme activity to cleave urokinase-type plasminogen activator (uPAR). The full-length isoform acts as a potent endothelial activator responsible for tumor progression. uPAR can be expressed by both endothelial and tumor cells (Andolfo et al. 2002). Laurenzana et al. (2014) demonstrated that EPCs transfected with a lentivirus encoding for MMP12 are recruited into melanoma mass under CXCR4/SDF1 system stimuli after intravenous delivery in experimental settings. Moreover, in vitro and in vivo, it was shown that MMP12-engineered EPCs reduced melanoma progression, intra-tumoral angiogenesis, and lung metastasis in old CD-1 nude mice, degrading uPAR on tumor cells and ECs (Laurenzana et al. 2014). Noteworthy, these ex vivo MMP12-engineered EPCs lost the capacity to perform capillary morphogenesis in vitro and, at the same time, acquired the antitumor and antiangiogenetic activity. Thus they seem to show no side effects in vivo (default pro-angiogenic role) (Duda et al. 2000). EPCs-based therapy using genetically transduced cells was also applied in a preclinical study for nasopharyngeal carcinoma. Wang et al. (2018) demonstrated that EPCs genetically modified with a lentiviral encoding for the metastatic gene suppressor KAI1/CD82 successfully inhibited lung metastasis in a nude mice bearing human nasopharyngeal carcinoma xenografts. However, there was little evidence regarding their potential to suppress the tumor cell graft.

A third EPCs-based strategy in cancer gene therapy is to accentuate the host immune system against cancer. For instance, Ojeifo et al. (2001) engineered EPCs to express IL-2 to stimulate natural killer and cytotoxic T cells in a syngeneic mouse model of melanoma lung metastases. They demonstrated that multiple intravenous injections abrogated the tumor metastases and prolonged animal survival. Muta et al. (2003) manipulated EPCs with a retrovirus vector carrying IL-12, showing that, in vivo, this gene therapy selectively delivers the protein to the tumor site in a xenograft rat model of breast cancer where its overexpression induced natural killer and cytotoxic T cells.

To overcome the controversies associated with the ESCs from human embryos, recently, iPSCs are considered the primary source of autologous or allogeneic pluripotent stem cells. They were explored as a source of human EPCs suitable as a delivery system of immune-stimulatory molecules to inhibit cancer. Purwanti et al. (2014) obtained $CD133^{+}/CD34^{+}$ EPCs from human iPSCs. They demonstrated that the cells expressed EPC-specific markers (i.e., CD31. VEGFR, cadherin) did not express hematopoietic cell markers (i.e., CD45), exhibited tubulogenesis in vitro, showed tumor tropism in an orthotropic lung metastasis mouse model for breast cancer, and did not enhance tumor growth and metastasis. Moreover, when these iPSCs-EPCs, engineered with a baculovirus encoding for the immune co-stimulatory molecule CD40 (with a pivotal role in the T-cell activation), were systemically injected in breast cancer-bearing mice, the animals showed prolonged survival (Purwanti et al. 2014). Noteworthy, in this study, an insect baculovirus was used instead of the conventional animal viral vectors. Insect virus bypasses the risk of virus replication and infection in the human host cells, and there is no host immune response (Bessis et al. 2004; Strauss et al. 2007). However, they are not adapted for long-term transgene expression.

A combination of suicide gene-targeting therapy with an antiangiogenic molecule was established in a human HepG2 liver cancer preclinical model to improve patients' treatment outcomes. Zhang et al. (2020) developed a gene therapy protocol with cytosine deaminase (CD) and endostatin gene transfected in EPCs obtained from fresh heart blood of adult BALB/c nude mice. Cytosine deaminase is one of the most widely investigated suicide gene/pro-drug that converts the nontoxic antifungal agent 5-fluorocytosine into the toxic chemotherapeutic agent 5-fluorouracil (5-FC) (Lawrence et al. 1998). The abovementioned preclinical model showed a total tumor volume reduction by MRI, angiogenesis inhibition visualized by VEGF- and CD31-positive immunostaining, decreased ECs, and increased tumor cell apoptosis assessed by TUNEL assay in mice transfected with CD/endostatin-EPCs plus 5-FC (intraperitoneally injected) compared to control treatment group (Zhang et al. 2020). CD/endostatin synergistic action could be translated in to clinical trials to target the hepatomas site via vein grafting.

Neoangiogenesis is mostly proven via CD31/VEGF immunohistochemistry, but this method is inadequate because it requires experimental animals to be sacrificed or human biopsies taken for immunohistological studies renders follow-up is impossible. Recently, studies are focused on advanced, noninvasive, and real-time molecular imaging methods as tracking strategies to monitor transplanted EPCs-based drug

vectors for antitumor therapy (Arbab et al. 2006). EPCs can be applied for noninvasive MRI investigation, as demonstrated by Chen et al. (2014a). They have approximately 100% of human PBMCs-derived EPCs efficiently labeled with N-alkyl–polyethylenimine 2 kDa (PEI2k)-stabilized superparamagnetic iron oxide (SPIO) nanoparticles. Moreover, functional assay outputs such as proliferation, migration, and tubulogenesis rates and incorporation into tumor neovasculature in vivo results have shown that these magnetic-labeled EPCs have the same activity as unlabeled ones. Once labeled, EPCs were intravenously or subcutaneously injected in a lung carcinoma xenograft model and were effectively detected by seven-tesla micro-MRI at the tumor site. The results showed excellent biocompatibility and magnetic resonance sensitivity even at a small alkyl-PEI2k/SPIO concentration than other contrast agents (Chen et al. 2014a). An EPC-based theranostic method has also been also proposed using the abovementioned MMP12-engineered EPCs radiolabeled with ^{111}In 8-oxyquinoline (oxine) for all the tumors displaying uPAR-dependent cancer progression (Laurenzana et al. 2014).

In glioma, the herpes simplex virus TK (HSV-TK)/ganciclovir (GCV) gene therapy is a suicide gene therapy widely used in both experimental and clinical trials thanks to its potent bystander effect (Zhang et al. 2010). A combination of HSV-TK suicide gene therapy with real-time molecular imaging has been reported for glioblastoma. Varma et al. (2013a) employed human cord blood-derived EPCs as a delivery vehicle for replication-competent adenovirus AD5 carrying both suicide genes, yeast CD (yCD) and mutant HSV-TK mutTK (SR39), and reporter gene, human sodium iodide symporter (hNIS) for I-131 (radioiodine) for diagnostic MRI imaging and single-photon emission computed tomography (SPECT). Their results indicated that AD5-yCD-mutTK-EPCs reached the glioma mass upon intra-tumor injection. Furthermore, double staining experiments demonstrated that both EPCs ($hNIS^+/vWf^+$) and tumor cells ($hNIS^+/EGFR^+$) expressed the transgenes thanks to the transfected EPCs' ability to deliver the vectors in the surrounding tumor cells (Varma et al. 2013a). Noteworthy, this study exploited intra-tumor injection instead of the prevalent systemic injection. The intra-tumor injection is advantageous to alleviate the virus's entry into circulation and curtail side effects (Lohr et al. 2001). Moreover, a replication-deficient virus is used as a transgenes delivery system to improve tumor cell death by their self-replication properties and infectivity of the surrounding cells in the vicinity (Barton et al. 2011; Barton et al. 2003). The reporter gene system with hNIS also overcame the short monitoring time (~7 days) with In-111Oxine labeling. hNISm allows repeated detection of the injected cells for extended periods (Barton et al. 2003; Varma et al. 2013a).

EPCs have also been proposed as the best vehicle to deliver therapeutic genes and imaging probe targeting glioma stem-like cells (GSCs) (Chen et al. 2014b). Glioma is a vascular-rich tumor with high resistance to antiangiogenic therapy because tumor cells can pass from the vascular phase of growth to the nonvascular one and vice versa. The mechanisms by which glioma achieve neovascularization are vascular co-option, angiogenesis, vasculogenesis, vascular mimicry, and GSCs-ECs transdifferentiation. GSCs-ECs transdifferentiation is implicated in the resistance against anti-VEGF therapy which currently in practice (Baisiwala et al. 2019;

Yan et al. 2017). Using in situ C6 glioma rat model, Chen et al. (2014b) showed that exogenous spleen-derived EPCs labeled with USPIO (ultra-small SPIO) integrate into the vessels containing glioma-derived ECs without inducing any promoting effect of GSCs transdifferentiation.

Despite these promising results, in vivo MRI with iron-nanoparticles presents some inconveniences: (i) the signal is lost over time due to the contrast agent biodegradation or dilution following cell division; (ii) time of EPC migration to the tumor site depends on multiple factors, such as tumor location and size, and chemotaxis factors expression levels; (iii) it is challenging to monitor real-time EPCs' migration into blood circulation; and (iv) imaging devices may lead to different results due to its sensitivity and resolution.

To enhance in vitro expanded EPCs translation from preclinical studies to clinical trials, the in vivo safety issues should be addressed because adverse effects and responses caused by EPCs therapy have been reported, such as collapse, sepsis, breast cancer development, and even death (Granton et al. 2015). In this context, Lee et al. (2019) proposed EPCs transplantation in dogs as a possible safety test of deleterious effects that should be conducted before EPCs application in human clinical trials. The choice of dogs lies in their physiological similarity with humans. They performed physical and laboratory examinations of human EPCs isolated from healthy donor PBMCs and transplanted intravenously into dogs. This in vivo safety assessment could be useful to test the minimal number of EPCs for transplantation because a high number is associated with pulmonary emboli or infarctions and affect immune responses (Beggs et al. 2006; Grigg et al. 1996; Prockop and Olson 2007).

Systemic delivery of EPCs gene therapy to primary tumors and metastases is the most attractive feature of using EPCs. Nevertheless, it remains to understand what factors permit EPCs persistence during hypoxia, migration, and proliferation to angiogenic sites, and if they are detained within the blood vessel wall or migrate further outside, and if they participate to vessel maturation.

Side Effects and Potential Risks of EPCs Cell Therapy

Given their advantages, EPCs are anticipated to play a pivotal role in cancer theranostics, for both therapy and biomarkers in future. However, various issues relevant to their use must be resolved before routine clinical use. New optimized stem cell differentiation protocols and animal models must be explored to better understand the molecular events involved in EPCs generation and differentiation. How to isolate and unequivocally identify the phenotype and functionality of EPCs remains problematic. The standardization of cell culture conditions, doses, and administration schedules will make it easier to understand different studies' results to interpret their data for future applications (Morales-Cruz et al. 2019).

EPCs are rare in both peripheral blood (0.01%) and bone marrow (0.05%). This necessitates their in vitro expansion to get them in large number for in vivo use. However, in vitro culture may alter their immunologic characteristics and tumorigenic potential. For example, during in vitro expansion, EPCs are exposed to

exogenous culture conditions different from physiological niches' microenvironment wherein stem cell proliferation and differentiation are under maintained under strict control. Consequently, EPCs could change their genome and phenotype that could render them tumorigenic thus contributing to tumor initiation. On the same note, sub-culturing will reduce stemness at every passage. This means that the development of new EPCs isolation methods is required to improve their yield as well as quality.

EPCs have a natural tropism for the sites of vascular injury. To avoid systemically adverse effects when used as delivery vehicles, advances in nanotechnology and tissue engineering are required to improve EPCs' homing-in and incorporation to the site of interest. When EPCs are employed to target drugs or genes to tumor cells, they could cause drug toxicity or drug resistance. For instance, after systemic injection, a small amount of them will reach the tumor site because most of them will be trapped in the lung, liver, or lymph nodes, causing therapeutic ineffectiveness and drug resistance (Brooks et al. 2018).

Another side effect could be a viral infection when viral carriers are used to genetically engineer EPCs (Goswami et al. 2019). Viral vectors currently employed in patient's treatment are classified as non-integrating or stable host-genome integrating vectors. The former include adenovirus and adenoassociated virus vectors and are primarily used for in vivo gene delivery in patients. Adenovirus can accommodate a large cDNA but are highly immunogenic. In contrast, adeno-associated viruses can only accommodate a smaller cDNA and are less immunogenic but retained for a longer time in the non-dividing cells. The last are retroviruses and lentiviruses. Both can harbor small cDNAs such as adeno-associated viruses, but unlike these, they allow for the prolonged-expression of the therapeutic gene, although there is a risk of insertional mutagenesis.

The evaluation of EPCs source for transplantation is essential. Allogeneic or autologous (via iPSCs technology) stem cell transplantation may provoke severe host immune responses or autoimmunity, respectively (Li et al. 2016b). Hematologic and lymphoid cancers are commonly treated by allogeneic HSC transplantation, but often patients incurred in Graft-versus-Host Disease (GVHD), acute or chronic, due to the induction of a complex immunological reaction of the donor's immunocompetent cells toward the recipient's tissues and organs. Different studies confirmed significantly improved outcomes, with a reduced incidence of chronic GVHD, but not acute one, after umbilical cord blood transplantation compared to allogeneic hematopoietic stem cell transplantation (Chen et al. 2017; Narimatsu et al. 2008).

It is necessary to consider the modulation of the host microenvironment as well. Cells and molecules of the microenvironment during hypoxia or increased inflammation have adverse effects on EPCs survival. For example, given the complexity and immunosuppressive properties of the tumor microenvironment, stem cell transplant combined with other therapies, such as immune checkpoint inhibitors, may better eliminate cancer and its recurrence. For example, Hu et al. engineered the surface of HSCs with the checkpoint inhibitor programmed death-1 (PD-1) antibodies-decorated platelets for the treatment of recurrent leukemia in mice (Hu et al. 2018).

A general recommendation is to pay attention when choosing EPCs as a therapy in oncology: it must be considered that mobilization and integration in tumor blood vessels depend on tumor type, stage, and treatment (Farnsworth et al. 2014). For example, cell therapies hold much more promise for treating diseases in which tissue can be ablated, such as bone marrow or skin cancers that can be easily removed with drugs or surgically, respectively. These procedures favor transplanted cells' engraftment because they will not have to compete with diseased resident cells. In more morphological complex tissues such as the brain, where massive ablation of diseased tissue is impossible, engraftment of transplanted cells is lower, and consequently, therapeutic efficacy is reduced.

Conclusion and Future Perspective

The chapter explores the underlying molecular mechanisms and potential applications of EPCs in cancer therapy. The chapter also discusses the protocols to obtain EPCs in a significant amount and their modification, administrated, besides the possible undesired effects and potential risks for cancer patients.

Despite that cancer is one of the leading public health problems, there are still no adequate and exhaustive therapeutic and diagnostic protocols available due to the incomplete knowledge of cancer cell biology. Among the various approaches for cancer theranostics, manipulated-stem cell transplant, alone or as an adjuvant for other therapies, could be a new strategy to treat cancer patients. Stem cells reside in almost all organs and tissues in the body, with the potential for self-renewal, migration, and differentiation that justifies their use in antitumor therapy. Therefore, studying stem cells for tissue engineering and theranostic resolutions is exciting. The existing results concerning stem cell therapy for cancer are highly encouraging. ESCs and iPSCs are the most powerful ones, but the diversity in their applications is still limited due to the possible risks related to viral vectors and ethics issues. EPCs are one of the autologous stem cell types for human use as they lack MHC-I expression, resistant to NK-mediated cytolysis, and primarily involved in blood vessel formation besides ease of availability and isolated and expanded ex vivo/in vivo, efficiently transduced to carry a therapeutic payload and home-in to the tumor and its vasculature. Hence, they are excellent cellular vehicles for systemic and local cancer therapy in general and angiogenic cancer in particular, as they primarily depend on blood vessels for growth and metastasis. It would be interesting to interfere with tumor vascularization by restoring a balance between pro- and antiangiogenic signaling and ensure direct access to drug delivery at the tumor site (Collet et al. 2016). Therefore, EPCs can be manipulated to selectively deliver the therapeutic molecules to the cancer cells while sparing the healthy cells. Given their biocompatibility and sensitivity when labeled, EPCs may serve as near-ideal vasculature tracker for diagnostic imaging.

Currently, only CEPCs are in clinical trials as surrogate biomarkers of antiangiogenic therapy. However, to validate the diagnostic value of CEPCs, the selection criteria of both cancer patients and healthy controls should be stricter due to the

Table 3 Summary of EPCs' advantages and disadvantages

EPCs versus other stem/progenitor cells in cancer therapy		
	Advantages	Disadvantages
Isolation	Less complicated	Low amount
Ex vivo expansion	Can generate enough cells for therapy	Massive tests must be performed to identify bona fide EPCs
Transduction	Efficient	
Tropism	Home to tumor proper and tumor vasculature	Only a minority of systemically administrated EPCs incorporate into tumor vessels
Drug	Protect the drug from inactivation	Systemically exposure
Immunological tolerance	Autologous EPCs not affected	

involvement of numerous confounding factors, that is, background cardiovascular diseases, diabetes mellitus, and lifestyles, which include smoking status and physical exercise, among others (Mayr et al. 2011).

Despite success in preclinical experimental animal models and enormous possibilities yet to be explored, the cell availability in small number, low-quality preparations, poor retention, low survival rate, and engraftment after transplantation still hamper EPC's routine clinical application (Sukmawati and Tanaka 2015; Terrovitis et al. 2010). Besides, there are no unique identifying markers for EPCs, and functional characterization of the rare putative EPC population-based on FACS phenotypes is challenging to realize for a large dataset. Hence, a consensus on the exact characterization and biology of EPCs is required to create a standardized, generally accepted methodology to develop the use of EPCs in clinical settings for regenerative approaches (Sabbah et al. 2019). Another drawback is the optimization of culturing protocols containing media without animals-derived supplements. Moreover, the establishment of stem cell-based anticancer therapies is slowed down by the lack of adequate financial support, the existence of ethical and political issues, and the easy authorization of new therapeutic protocols for which the efficacy has not been adequately tested. The current gap between public expectancy and actual progress of stem cell-based therapies in the clinical threatens regenerative medicine's social license to operate (Cossu et al. 2018). A possible step forward is to develop a combinatorial approach on several fronts (tumor vasculature, tumor cell tumor microenvironment, immune system) to achieve a better outcome. In conclution, EPCs translation from bench to antitumor therapy and diagnostic imaging depends on a more in-depth assessment (Table 3).

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Non-cryopreserved Peripheral Stem Cell Autograft for Multiple Myeloma and Lymphoma in Countries with Low Resources

48

Mohamed Amine Bekadja

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Abstract

Autologous peripheral blood progenitor cell (PBPC) transplant is a standard indication in the multiple myeloma (MM) and lymphoma. The use of non-cryopreserved PBPCs is not usual despite its safety, feasibility, and efficacy. Few data exists in the literature regarding the procedures for non-cryopreserved autologous PBSCs transplant in countries with limited resources. The bibliographical research of this work was limited on sites like PubMed, Google scholar; using the following articles on the non-cryopreserved autologous PBPCs in hematological malignancies in developing

M. A. Bekadja (✉)
Department of Hematology and Stem Cell Therapy, Etablissement Hospitalier Universitaire 1st November, Ahmed Benbella 1 University, Oran, Algeria

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countries have been selected. These papers were analyzed in terms of mobilization, apheresis, preservation and viability, conditioning regimen, engraftment, response, and finally survival. This chapter sums up experience from 17 transplant centers which carried out autografts with non-cryopreserved PBPCs in 1541 patients suffering from hematologic malignancies. The results in terms of mobilization showed a median CD34+ = 3.94 × 10^6/kg (range, 0.32–24.6), a viability >90% and >75% respectively in MM and lymphomas after a conservation of 24–144 h at +4 °C. The engraftment (mean neutrophils = 12.38 (6–86) days, mean platelets = 15.57 (7–89) days, and TRM (4.39%) were very satisfactory. In conclusion, this method is easy, efficient, and safe, and it is expected to grow in developing countries due to its low production cost and procedure simplicity.

Keywords

Cryopreserved · G-CSF · Hematopoietic · Progenitor · Stem cells · Transplantation

List of Abbreviations

AfBMT	African Blood and Marrow Transplantation
ASCT	Autologous stem cell transplantation
BEAM	BCNU-Etoposide-Aracytine-Melphalan
BM	Bone marrow
BMPCs	Bone marrow progenitor cells
CBV	Cyclophosphamide-BCNU-Etoposide/VP16
CEC	Cyclophosphamide, Etoposide, Carboplatin
DFS	Disease-free survival
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulfoxide
EMBMTG	Eastern Mediterranean Bone Marrow Transplantation Group
G-CSF	Granulocyte-colony stimulating factor
HPCs	Hematopoietic progenitor cells
LK	Leukapheresis
MEL/VP16	Melphalan, Etoposide
NE	Neutrophil engraftment
OS	Overall survival
PBSCs	Peripheral blood stem cells

Introduction

Cryopreservation of hemopoietic stem cells was established in the early 1970s (Gorin and Duhamel 1975, 1978; Gorin et al. 1978a, b; Gorin 1986; Douay et al. 1982) on bone marrow (BM) collected from iliac spines and stored usually at −140 °C or −196 °C in vapor or liquid phase nitrogen. Efficient cryopreservation of stem cells opened the way to autologous stem cell transplantation (ASCT) after high-dose chemotherapy and/or

total body irradiation. Since the early 1990s, peripheral blood stem cells (PBSCs) collected by leukapheresis (LK) have replaced bone marrow as a source of hemopoietic stem cells in almost 95% of the patients (Storb et al. 1977).

Cryopreservation of stem cells requires facilities, equipment for programmed freezing, and liquid nitrogen availability for storage with temperature monitoring. It also requires quality controls at thawing to ensure good quality cell preparation (Haider 2017). All steps are laborious, time-intensive, and expensive to ensure clinical grade quality of the cell preparation (Gorin 1986).

Several attempts have been made to assess alternative strategies to avoid cryopreservation, including maintaining the autograft at 4 °C in a conventional refrigerator. It has been rapidly shown that the viability of marrow stem cells cannot be preserved beyond 56 h (Burnett et al. 1983). On the contrary, PBSCs are more purified, devoid of erythrocytes and neutrophils, and can be easily preserved for extended periods. Several teams have demonstrated the possibility of using non-cryopreserved PBSCs to autograft patients with multiple myeloma who received high dose Melphalan over 1 or 2 days, with a preservation duration not exceeding 3 days (Wannesson et al. 2007; Lopez-Otero et al. 2009; Kayal et al. 2014; Bekadja et al. 2017; Bittencourt et al. 2019; Bekadja et al. 2012).

Patients with lymphomas autografted with PBSCs receive pretransplant regimens, such as the BEAM (BCNU-Etoposide-Aracytine-Melphalan), TEAM (as BEAM but Thiotepa instead of BCNU), or CBV (Cyclophosphamide-BCNU-Etoposide/VP16) over longer periods, lasting up to 6 days. Some studies show promising results with non-cryopreserved PBSCs kept at 4 °C for 3–6 days (Sierra et al. 1993; Karduss-Urueta et al. 2014). Even a few studies have claimed better results with non-cryopreserved as compared to the cryopreserved PBSCs (Sarmiento et al. 2018; Bittencourt et al. 2019).

Autologous bone marrow progenitor cells (BMPCs) or PBPCs provide a supportive therapy that allows the use of high doses, intensive antitumoral chemotherapy in hematological malignancies (Kessinger et al. 1986, 1988). Many studies have shown the superiority of autologous BMPCs or PBPCs over the conventional chemotherapy in hematological malignancies, such as multiple myeloma (MM) (Fermand et al. 2005) or lymphoma (Philip et al. 1995; Andre et al. 1999; Carella et al. 2009). PBPCs are currently used in autologous stem cells transplantation in hematological malignancies only, and BMPCs are reserved to specific indications, such as haploidentical allografts, or bone marrow failure. PBPCs are typically cryopreserved in liquid nitrogen at −180 °C in dimethyl sulfoxide (DMSO) and albumin (Billen 1957; Bakken 2006; Berz et al. 2007). Progenitor cells should be washed and cleaned from DMSO before use in the patient. This preservation technique requires expensive equipment. In vitro study concerning the use of non-cryopreserved hematopoietic progenitor cells (HPCs) was first published in 1957 (Billen 1957). It was then followed by studies on the conservation of PBPCs at +4 °C (Ahmed et al. 1991; Sierra et al. 1993; Preti et al. 1994) and their clinical use (Hechler et al. 1996). Very few autologous stem cells transplant were performed with non-cryopreserved PBSCs, and in our opinion, there are no published randomized or controlled studies on the non-cryopreserved autologous PBPCs. Only two literature

reviews on this topic have been published. The first study was published by Wannesson et al. in 2007 on autologous HPCs transplantation (bone marrow and peripheral blood) in hematological malignancies and solid tumors (Wannesson et al. 2007) while the second study was published by Al-Anazi in 2012, on the use of autologous PBPCs for transplantation in the patient with MM (Al-Anazi 2012).

This chapter reports all the published data in the field of non-cryopreserved autologous transplantation in hematological malignancies in developing countries to show the clinical safety, feasibility, and efficacy of the non-cryopreserved autologous cells to promote this technology, especially in the countries with limited resources.

Bibliographic Literature Search for the Chapter

Bibliographic research was based on PubMed and Google scholar, using the following keywords: hematopoietic, progenitor, non-cryopreservation, and autograft. All the articles on the non-cryopreserved autologous PBPCs in hematological malignancies in developing countries were searched. The selected papers were analyzed in terms of mobilization, apheresis, storage and viability, conditioning regimen, engraftment, response, and finally, survival. Data from this review were synthesized in a descriptive manner. This included the tabulation of study characteristics and outcomes. All the survival times were calculated from the date of transplant. Transplant-related mortality (TRM) was defined as any death related to a fatal complication in the absence of the underlying disease within 100 days from transplantation. Overall survival (OS) was defined as the duration from the date of transplantation until the date of death or the date of follow-up when the patient was known to be alive. Progression-free survival (PFS) was calculated from the date of transplantation to disease progression or death (regardless of the cause of death). The OS and the PFS were determined using the Kaplan–Meier estimation with 95% confidence intervals from standards errors.

Data Analysis from the Selected Studies

Research on PubMed and other search engines identified several publications and abstracts. In developing countries, only 17 studies were selected, responding to the criteria concerning non-cryopreserved autologous progenitor cells transplant. Countries of origin are by alphabetical order: Algeria, Brazil, Chile, Colombia, Egypt, Greece, India, Iran, Mexico, Morocco, and Thailand. All the 17 studies published from 2000 to 2021 were retrospective of "single-center case series" type. The majority focused on the MM and lymphoma (Papadimitriou et al. 2000; Ruiz-Arguelles et al. 2003; Cuellar-Ambrosi et al. 2004; Mabed et al. 2006; Lopez-Otero et al. 2009; Ramzi et al. 2012a, b; Kayal et al. 2014; Bekadja et al. 2017, 2021; Sarmiento et al. 2018; Kardduss-Urueta et al. 2018; Naithani et al. 2018; Kulkarni et al. 2018; Bittencourt et al. 2019; Jennane et al. 2020; Piriyakhuntorn et al. 2020) and only one study focused on acute leukemia (Palumbo et al. 2009).

Over 21 years, a total of 1541 autografts were performed with unfrozen stem cells. The results of these autografts are detailed according to the different stages of the autograft, which are mobilization, apheresis, storage, viability, conditioning regimen, engraftment, and posttransplantation results. A special section is reserved for comparison of the studies between frozen and unfrozen stem cells.

Cell Mobilization

Most centers have performed PBPCs mobilization using granulocyte-colony stimulating factor (G-CSF) alone, while two groups have used G-CSF combined with chemotherapy. In multiple myeloma, all studies (Ruiz-Arguelles et al. 2003; Mabed et al. 2006; Palumbo et al. 2009; Bekadja et al. 2012, 2017; Ramzi et al. 2012b; Kayal et al. 2014; Bittencourt et al. 2019; Jennane et al. 2020) have conducted the mobilization with subcutaneous G-CSF alone at a dose of 15 μg/kg/day, or 5 μg/kg twice a day, for 4–5 consecutive days. In lymphomas, mobilization was performed using G-CSF (5 μg/kg/day for 3 days) in combination with cyclophosphamide at a dose of 1.5 g/kg/day, for 3 days, in two groups (Mabed et al. 2006; Lopez-Otero et al. 2009).

Apheresis

The PBPCs apheresis was performed using devices such as Haemonétics®, Cobe Spectra®, or Optia®. Leukapheresis was started as soon as the flow cytometer counting of CD34+ (cluster differentiation) PBSCs was greater than 1×10^6cells/μl. The mean number of leukapheresis was two in MM and three in lymphomas. The overall mean of CD34+ collected in all studies was 3.94×10^6/kg (range, 0.32–24.6). In MM, the overall mean of CD34+ collected was 4.26×10^6/kg (range, 0.32–27.8). It was 4.47×10^6/kg (range, 1.9–24.6) in lymphomas. There was no report of mobilization failure in the published series.

Cell Storage

The PBPCs collected were saved in the refrigerator at +4 °C for a period ranging from 1 day to 6 days (Sierra et al. 1993; Hechler et al. 1996; Bekadja et al. 2017, 2021) depending on the type of conditioning regimen used. In the MM, storage time ranges from 1 to 2 days, and it was of 3–6 days in lymphoma (Table 1).

Cell Viability

The viability of PBPCs was calculated by the Trypan Blue technique and by flow cytometry (Fleming and Hubel 2006). The average viability was over 90% in MM and over 75% in lymphomas.

Table 1 Patient's characteristics and results of non-cryopreserved autologous peripheral blood progenitor cell transplantation

Author	Patients (n)	Age (year)	Diagnosis	HDCT	CD34+ reinfused	Storage at +4 °C (hours)
Papadimitriou (2000)	72	8–69	MM/NHL/HL	Mel140-180/Mel-VP16	3(0.8–2.78)	24–60 h
Ruiz-Arguëlles (2003)	46	9–67	MM/NHL/HL/AML/ALL	Mel 200	4.68	24–72 h
Cuellar-Ambrosi (2004)	47	12–67	MM/NHL	CBV/CTX-TBI/Mel 200	1.36 (0–6.32)	144 h
Mabed (2006)	28	16–50	HL	CTX/VP16/Carboplatin	6.4 (3.8–24.6)	72 h
Mabed (2006)	32	17–55	NHL	CBDA/VP16/CTX	>3	72 h
Lopez-Otero (2009)	26	42–66	MM	Mel 200	7.6 (0.3–14.8)	24 h
Ramzi (2012)	45	NA	HL	CEAM	3.4	NA
Ramzi (2012)	38	NA	MM	Mel140/Mel200	NA	48 h
Kayal (2014)	92	22–65	MM	Mel 200	2.9 (0.9–7.67)	48 h
Bekadja (2017)	240	35–65	MM	Mel140/Mel200	5.7 (1.9–10.5)	24 h
Sarmiento M (2018)	42	22–68	MM/NHL/HL	Mel 200	5.1 (2.5–5.6)	
Kardduss-Urueta et al. (2018)	359	54 (29–75) 39 (5–67)	MM/Lymphoma	Mel200/BEAM/CBV	3.6	72 h (48 h–6 days)
Naithani (2018)	76	59 (34–68) 34 (14–64)	MM Lymphoma	Mel200/140 BEAM	2.56 (1.2–7.9)	48 h–6 days
Kulkarni (2018)	224	50 (23–68)	MM	Mel200	4.87	24–48 h
Jennane (2020)	55	37–67	MM	Mel140/Mel200	4.5 (2–12.2)	24–48 h
Piriyakhuntorn (2020)	26	55.7	MM	Mel200	3.8 (2.0–16.5)	48 h
Bekadja (2021)	94	29 (17–60)	HL/NHL	CBV/BEAM/BeEAM/EAM	4.12 (3.4–5.4)	6 days

Abbreviations: *HDCT*, high dose chemotherapy; *MM*, multiple myeloma; *NHL*, non-Hodgkin lymphoma; *HL*, Hodgkin lymphoma; *AML*, acute myeloid leukemia; *ALL*, acute lymphoid leukemia

Conditioning Regimen

The conditioning regimen and the myeloablative therapy were dependent on the diagnosis. In MM, all of the studies have used the Melphalan at a dose of 180 (Papadimitriou et al. 2000) or 200 mg/m^2 on D-1 (Lopez-Otero et al. 2009; Ramzi et al. 2012b; Kayal et al. 2014; Bekadja et al. 2017). In lymphoma, the protocols used were of a different type: MEL 200 (Melphalan 200 mg/m^2), CBV (Lobo et al. 1991), BEAM (BCNU, Etoposide, Aracytin, Melphalan) (Smith and Sweetenham 1995), CEAM (Lomustine, Etoposide, Aracytin, Melphalan), EAM (Etoposide, Aracytin, Melphalan) (Bekadja et al. 2018), CEC (Cyclophosphamide, Etoposide, Carboplatin), MEL/VP16 (Melphalan, Etoposide), and their length varies from 3 to 6 days (Table 2).

Table 2 Results of engraftment with non-cryopreserved autologous peripheral blood progenitor cell transplantation

Author	Patients (n)	Diagnosis	Neutrophils >0.5G/L (median day and range)	Platelet>20G/L (median day and range)	TRM (%)	Graft failure patients
Papadimitriou (2000)	72	MM/NHL/HL	9 (6–16)	5 (0–89)	0	0
Ruiz-Argüelles (2003)	46	MM/NHL/HL/AML/ALL	14 (0–86)	25 (0–102)	2	0
Cuellar-Ambrosi (2004)	47	MM/NHL	11 (9–15)	16 (11–44); 15 (14–20)	12.7	0
Mabed (2006)	28	HL	13 (7–18)	15 (7–20)	–	0
Mabed (2006)	32	NHL	12 (8–17)	14 (7–19)	9.37	0
Lopez-Otero (2009)	26	MM	27 (0–53)	37 (0–73)	9.6	0
Ramzi (2012)	45	HL	11	14	2.2	0
Ramzi (2012)	38	MM	11 (9–21)	13 (10–31)	0	0
Kayal (2014)	92	MM	10 (8–27)	14 (9–38)	3.2	0
Bekadja (2015)	45	HL	11 (8–12)	13 (10–24)	3	0
Bekadja (2017)	240	MM	10 (6–17)	13 (9–24)	1.3	0
Sarmiento (2018)	42	MM/NHL/HL	9 (9–16)	11 (10–19)		0
Kardduss-Urueta et al. (2018)	359	MM, Lymphoma	13 (9–39)	16 (7–83)		0
Naithani (2018)	76	MM, Lymphoma	12 (9–35)	13 (9–65)	3	0
Kulkarni(2018)	224	MM	12 (9–22)	17 (10–44)	3.1	1/224
Piriyakhuntorn (2020)	26	MM	12 (10–19)	14 (10–23)	3.8	0
Jennane (2020)	55	MM	12 (7–19)	14 (9–32)	3.6	0
Bekadja (2021)	94	HL/NHL	14 (12–32)	17 (15–28)	9	0

Table 3 Survival of patients with multiple myeloma and lymphoma autografts with non-cryopreserved PBPCs

Author	Patients number	Diagnosis	Follow-up (median months)	OS	PFS
Mabed (2006)	28	HL	16	45% at 24 months	42% at 24 months
Mabed (2006)	32	NHL	18	50% at 24 months	43% at 24 months
Lopez-Otero (2009)	26	MM	NA	80% at 76 months	NA
Ramzi (2012)	45	HL	27	27 months (median)	20 months (median)
Ramzi (2012)	38	MM	31	30 months (median)	27 months (median)
Bekadja (2017)	240	MM	47	79% at 5 years	59 months
Kulkarni (2018)	224	MM	NA	62.9% at 5 years	36.6% at 5 years
Piriyakhuntorn (2020)	26	MM	37.5	NR	16 months
Bekadja (2021)	94	NHL/HL	62	85% at 36 months	70% at 36 months

Abbreviations: *MM*, multiple myeloma; *HL*, Hodgkin lymphoma; *NHL*, non-Hodgkin lymphoma; *OS*, overall survival; *PFS*, progression-free survival; *NA*, nonavailable; *NR*, not reached

Cell Engraftment

Engraftment was defined by the rate of ANC (absolute neutrophil count) over 0.5 G/L and a platelet count greater than 20 G/L, except for one study in which the threshold was 25 G/L platelets (Ruiz-Arguelles et al. 2003). The results of engraftment in the different studies are shown in Table 3. The overall mean recovery time of ANC was 12.38 days (range, 6–86), and that of the platelets was 15.57 days (range, 7–89). This recovery time was respectively 13 and 18 days in MM and 12 and 14 days in lymphoma. There was no engraftment failure recorded among the different studies, only one study (1/224) (Kulkarni et al. 2018).

The overall median rate of the transplant related mortality (TRM) was 4.39%, while it was 3.51% and 4.85% respectively in the MM and lymphoma.

Posttransplant Data

Considering the heterogeneity of the studies in terms of diagnosis and intensification protocols, it is difficult to analyze them in relation to response or survival. However, the median follow-up period ranged between 31 and 37.5 months in the MM, and between 16 and 62 months, in lymphoma. The overall survival (OS) and disease-free survival (DFS) in the MM and lymphoma are reported respectively in Table 4.

Table 4 Characteristics of patients receiving PBSC stored at 4Dg C in Oran and pair matched patients receiving cryopreserved PBSC reported to the EBMT registry

PBSC	Stored at 4 °C[a]	Cryopreserved[b]	P value
Number of patients	94	351	–
Age at SCT			
Median (range)	29 (17–60)	29 (17–63)	0.90
IQR	25–34	24–35	
Gender (%)			
Female	46 (49)	169 (48)	0.98
Male	48 (51)	182 (52)	
Karnofsky at SCT (%)			
Good ≥80	79 (84)	309 (88)	
Poor <80	13 (14)	34 (10)	0.49
Unknown	2 (2)	8 (2)	
Time from transplant to SCT (months) median			
Range	11	12	
IQR	3–85	3–89	0.65
	9–16	9–16	
Lymphoma type (%)			
Mantle cell	4 (4)	16 (5)	
DLBCL	18 (19)	67 (19)	
Hodgkin	66 (70)	252 (72)	0.92
MZL	1 (1)	3 (1)	
T-cell	5 (5)	13 (4)	
Disease status at SCT			
CR	70 (75)	267 (76)	
PR	19 (20)	64 (18)	
PIF	1 (1)	4 (1)	0.97
Relapse	4 (4)	16 (5)	

Abbreviations: *SCT: stem cell transplantation; DLBCL: diffuse large B cell lymphoma; MZL: marginal zone lymphoma; CR: complete remission; PR: partial remission*
[a]Patients from Oran
[b]Patients from the LWP/EBMT Registry

Comparative Studies

There are very few studies comparing the use of frozen and unfrozen stem cells during autografts. The results of the significant studies listed have been described in the following sections.

The Study by Sarmiento et al. (2018)

In total, 111 auto-HSCT were performed from January/2015 to October/2016 (42 non-cryopreserved and 74 cryopreserved). There were 69 patients who had the underlying diagnosis of MM. The main objective of the study was to compare early hematological and non-hematological complications after auto-HSCT between both groups of patients, measured by: infusion reactions on day 0, incidence and grade of

oropharyngeal and gastrointestinal mucositis and the use of high dose morphine, incidence of febrile neutropenia, hematopoietic recovery after auto-HSCT, and length of hospital stay between non-cryopreserved group and cryopreserved group. The number of CD34+ cells collected, and cell viability between both groups of patients were also compared. No differences were observed in the characteristics of the apheresis products and their viability.

Engraftment was significantly faster in the non-cryopreserved group ($p = 0.001$). Febrile neutropenia and severe mucositis were lower in the non-cryopreserved group (40% vs. 92% $p = 0.0001$ and 11% vs. 64%, $p = 0.001$, respectively). In addition, length of hospitalization was 5 days shorter in the non-cryopreserved group ($p = 0.0001$). Mean hospital stay was 20 days (range 14–54) in the cryopreserved group versus 15 days (range 9–20) in the non-cryopreserved group ($p = 0.0001$). Overall responses and transplantation outcomes were similar.

The Study by Bittencourt et al. (2019)

A total of 108 ASCTs were included in two groups: 63 in cryopreserved and 45 in non-cryopreserved. Patients in cryopreserved were mobilized with G-CSF alone, Cyclophosphamide+G-CSF, or G-CSF + Plerixafor. In non-cryopreserved, all patients were mobilized with G-CSF alone. A minimum yield of 2×10^6CD34+ cells/kg was mandatory to undergo ASCT. For cryopreservation, 10% DMSO was added and the product stored in mechanical freezer at −80 °C. Non-cryopreserved cells were stored in blood bank refrigerator at 4 °C for a maximum of 48 h. All patients were conditioned with Melphalan (200 mg/m^2 or 140 mg/m^2, if chronic renal failure or heart failure).

The only statistical difference was the dose of CD34+ cells/kg, slightly higher in cryopreserved (4.00×10^6 (IQR: 3.20–5.10) vs. 3.50×10^6 (IQR: 2.80–4.60), $p = 0.04$). Neutrophil engraftment (NE) was significantly delayed in cryopreserved: median of 13 days (IQR: 12–15) as compared with 10 days (IQR: 10–11) in non-cryopreserved ($P < 0.0001$); and it remained delayed in cryopreserved regardless of the time of transplantation. Graft failure occurred in one patient (cryopreserved); extended follow-up showed NE on day +35. The early TRM rate was 5% (three patients) in cryopreserved and 2% (one patient) in non-cryopreserved. In the first group, two patients died before NE, days +26 and +28 post-ASCT, due to fungal infection; the third died on day +20 from acute respiratory insufficiency of unknown cause, having engrafted on day +13. In the second group, a patient engrafted on day +10 and died on day +18 from septic shock. Infection and neutropenic fever occurred with no statistical difference in cryopreserved and non-cryopreserved (41% vs. 29% infection, $p = 0.23$; 41% vs. 38% neutropenic fever, $p = 0.84$, respectively) and were not associated with delay in NE. Cost analysis revealed a total of $1300 for one cryopreserved unit and $300 for one non-cryopreserved unit.

The Study by Bekadja et al. (2021)

Recently, we published results comparing autografts with unfrozen cells from our center with those from the EBMT group using frozen cells in lymphomas

(Bekadja et al. 2021). We compared outcomes of 94 consecutive adult patients with lymphoma (66 with Hodgkin lymphoma) autografted in our department in Oran (Algeria) using PBSCs stored at 4 °C, from 2009 to 2018, with patients receiving cryopreserved stem cells reported to the European Society for Blood and Marrow Transplantation registry. The 94 patients autografted in Oran were matched to 351 EBMT patients receiving cryopreserved PBSCs in the registry (four controls per patient in Oran). These data showed higher CD34 + levels with unfrozen cells, neutrophil engraftment was significantly faster with cryopreserved PBSCs ($p = 0.003$). Likewise, platelet recovery to 20,000/mm^3 was significantly faster in patients receiving cryopreserved PBSCs ($p = 0.01$). However, all patients in both groups had recovered by day 20. For non-cryopreserved versus cryopreserved PBSCs, the non-relapse mortality was 9% versus 7% ($P = 0.4$), relapse incidence was 22% versus 32% ($p = 0.13$), PFS at 36 months was 70% versus 61% ($p = 0.4$), and overall survival was 85% versus 75% ($P = 0.3$). There were no significant differences in non-relapse mortality ($p = 0.4$), relapse incidence ($p = 0.13$), progression-free survival ($p = 0.4$), or overall survival ($p = 0.3$) (Figs. 1 and 2), suggesting that the impact of conditioning does not play a significant role in comparing the two groups. It was concluded that in patients with lymphoma receiving pretransplant regimens, such as BEAM, PBSCs stored at 4 °C for up to 6 days could be used safely in centers with no cryopreservation facility.

The Study by Garifullin et al. (2021)

A retrospective analysis of multiple myeloma patients who underwent autologous transplantation using non-cryopreserved and cryopreserved grafts were performed from March 2016 to April 2020. There were no differences in the total number of CD34 + cells × 10^6/kg. The adverse events were absent in the non-cryopreserved group. But 29/43 (67.4%) patients had such symptoms in the cryopreserved group on day 0 (nausea and vomiting – 7 patients (16.3%), tachycardia – 16 patients (37.2%), stenocardia – 3 patients (7.0%), arterial hypertension – 6 patients (13.9%), increased total bilirubin and indicator liver enzymes – 5 patients (11.6%)). All patients had full recovery of hematopoiesis till discharge from the hospital. Neutrophil recovery was achieved on the 11th day (range 9–14) and platelets on 12th day (range 8–19) in the non-cryopreserved group, and the 10th day (range 8–14) and 12th day (range 8–20) in the cryopreserved group, respectively. The authors concluded that this method of storage of PBSCs without cryopreserved was equal to the traditional method of controlled freezing with dimethyl sulfoxide and can be used in hospitals that have no cryobank in their composition.

An In-Depth Analysis of the Published Data

PBPCs autologous transplant is indicated as first-line treatment in the MM (Rajkumar 2011; Moreau et al. 2011; Tosi et al. 2012), in the mantle cell lymphoma (Salek et al. 2014), as consolidation treatment in the diffuse large cell lymphoma (Redondo et al. 2014; Shin et al. 2014), and as salvage therapy during relapsed or

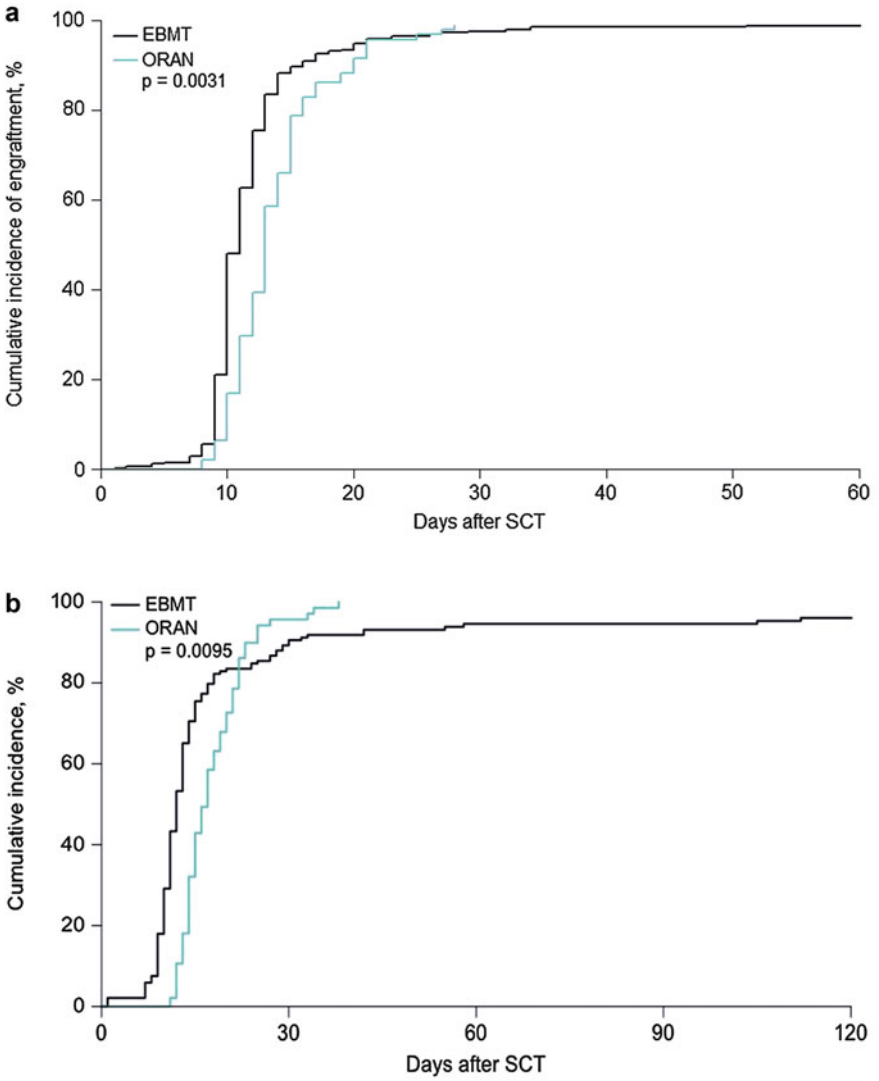

Fig. 1 Kinetics of engraftment of patients autografted with PBSC stored at 4 °C and patients autografted with cryopreserved PBSC. (**a**) Cumulative incidence of sustained neutrophil recovery >500/uL: Significant faster engraftment with cryopreserved PBSC ($p = 0.003$). (**b**) Cumulative incidence of sustained platelet recovery >20,000/uL. Significant faster engraftment with cryopreserved PBSC ($p = 0.01$)

refractory forms of Hodgkin's diseases (Hahn et al. 2013; Isidori et al. 2013; Van Den Neste et al. 2013) or non-Hodgkin lymphomas (Milpied 2013).

A recent EBMT survey published in 2021 shows 48,512 stem cell transplants in 43,581 patients, including 28,714 autografts (59%). The EBMT group had

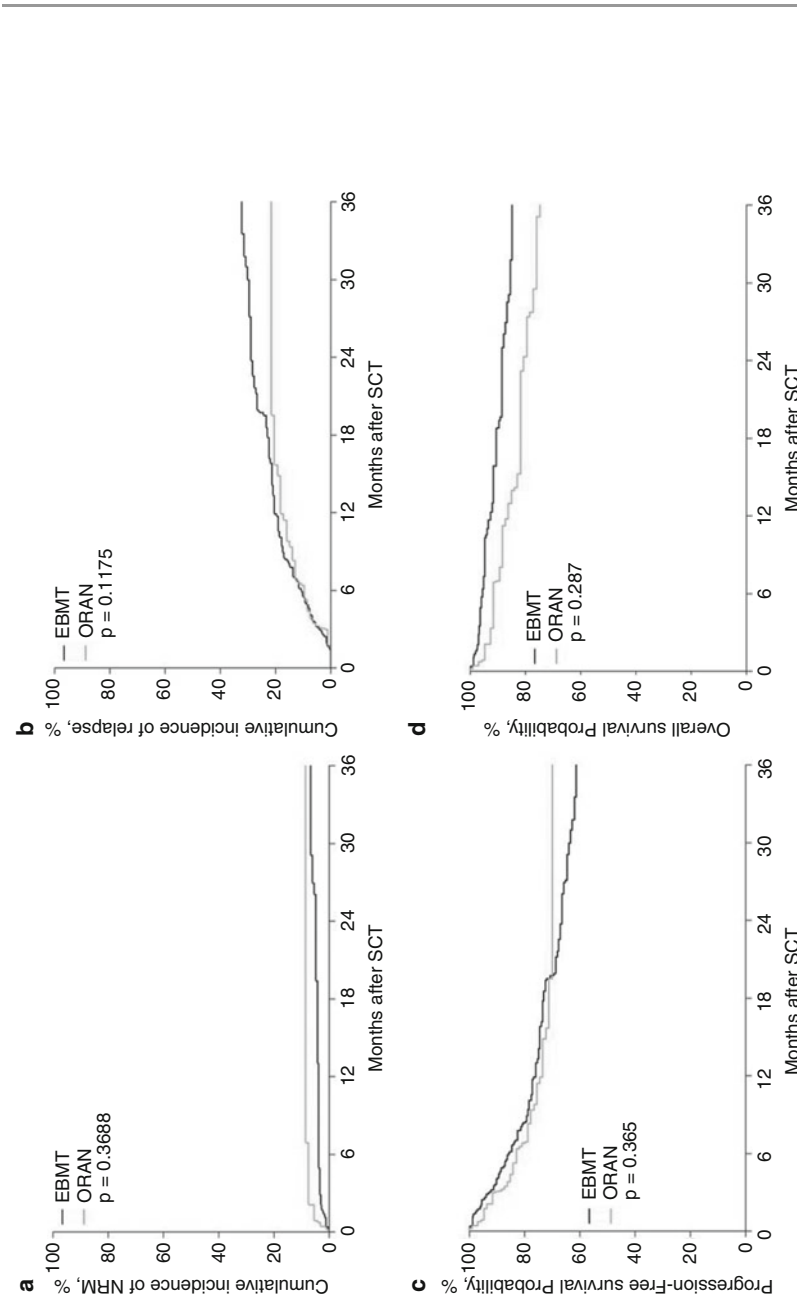

Fig. 2 Outcome of the patients autografted with PBSC stored at 4 °C and patients autografted with cryopreserved PBSC: No significant difference in any parameter. (**a**) Non-relapse mortality; (**b**): relapse incidence; (**c**): progression-free survival; and (**d**) overall survival

700 centers in 51 countries in 2019. These European centers freeze stem cells as part of the autografts.

The Eastern Mediterranean Bone Marrow Transplantation Group (EMBMT) represents the Eastern Mediterranean Region (EMRO) comprising 10 countries, including Algeria, Morocco, Tunisia, Egypt, Lebanon, Iran, Saudi Arabia, Pakistan, Jordan, and the Sultanate of Oman. The 2008–2009 report has shown an activity rate of autografts of 36.5% ($n = 483/1322$ total first transplants) in these countries, versus 59% ($n = 16{,}591/28033$ total first transplants) in the developed-country (Mohamed et al. 2011). Most transplant centers do not use stem cell freezing during autologous transplantation.

The report also highlights the limited number of transplant centers in developing countries which is 14 versus 647, i.e., 46 times greater for 2009, in developed countries (Baldomero et al. 2011). Moreover, the average number of transplant center is 14/country in the EBMT Group, which counts 48 countries in 2009 versus 1.4/country in the EMBMT group which has 10 countries. The number of centers per 10 million inhabitants is respectively 7.6 and 0.3 in developed countries versus developing countries. Moreover, the number of transplants per 10 million inhabitants is 467 and 28.7 in the EBMT and EMBMT Groups, respectively. Hence, this situation showed that new transplant centers and particularly the development of the non-cryopreserved autologous PBPCs transplant in these countries and other resource-constrained countries were necessary.

The first meeting of the African Blood and Marrow Transplantation (AfBMT) was held in Casablanca from April 19, 2018, to April 21, 2018, to foster HSCT activity in Africa. Out of the 54 African countries, HSCT is available only in 6 (Algeria, Egypt, Morocco, Nigeria, South Africa, and Tunisia) (Mhamed Harif 2019).

The latest published report of WBMT (Baldomero et al. 2019) showed that AFR/EBMT region represented only 3% of reported activity worldwide. In the African continent, this is even lower. The health care systems in Africa are the least developed.

Hematopoietic cell transplant activity in the 28 countries comprising Latin America is poorly defined. A retrospective study conducted a voluntary survey of members of the Latin American Bone Marrow Transplantation Group regarding transplant activity 2009–2012. Annual activity increased from 2517 transplants in 2009 to 3263 in 2012 represents a whopping 30% increase. The median transplants rate (transplant per million inhabitants) in 2012 was 64 (autotransplants, median 40; allotransplants, median 24). This rate is substantially lower than that in North America and European regions (482 and 378) but higher than in the Eastern Mediterranean and the Asia Pacific regions (30 and 45). However, the Latin America transplant rate is five to eightfold lower than in America and Europe, suggesting a need to increase transplant availability. Transplant team density in Latin American (teams per million population; 1.8) is three to fourfold lower than that in North America (6.2) or Europe (7.6) (Jaimovich et al. 2017).

The number of publications regarding non-cryopreserved autologous transplant is scarce because of the widespread use of cryopreserved stem cells. In this chapter,

only 17 eligible studies were collected dealing with non-cryopreserved autologous transplant in hematological malignancies (lymphoma and MM) in some developing country, from 2000 to 2020. All these studies are single-center, retrospective, non-randomized, and uncontrolled, reflecting the care of patients in real life and working conditions of countries with limited resources. Only one study is prospective and included 26 patients with MM (Lopez-Otero et al. 2009). Currently, over 1500 patients have undergone autologous transplantation with unfrozen cells, out of which 80% are relevant to multiple myeloma while the remaining 20% are concerning to lymphoma.

Cell Mobilization

The first step of autologous stem cells transplantation is PBPC mobilization. There is no absolute rule in PBPC mobilization, but multiple studies have been published regarding recommendations for improving the harvesting efficiency of PBPCs (Bezwoda et al. 1994; Demirer et al. 1996). Two main methods are used in PBPC mobilization; the first relates to the use of growth factor G-CSF alone, given subcutaneously at a dose of 10 to 15 μg/kg/day or 5 μg/kg twice a day, for 5 days (Talhi et al. 2013; Yafour et al. 2013); the second consists in the combination of G-CSF with chemotherapy (Ford et al. 2006). There have been not much difference in the performance of harvesting of CD34+, but the second method requires hospitalization to manage aplastic anemia secondary to chemotherapy, whereas in the first method, the use of G-CSF alone can be done at home, which reduces the costs of the autograft procedure. Most studies have achieved mobilization with G-CSF alone, especially in the MM and lymphoma; only two groups have used G-CSF in combination with cyclophosphamide (Mabed and Al-Kgodary 2006; Mabed and Shamaa 2006). The objective of the mobilization is to reach at least 2×10^6/kg CD34+ in the MM in which the conservation of CD34+ is short (24–48 h) and at least 4×10^6/kg in lymphomas in which the conservation of CD34+ is most extended (3–6 days). Indeed, the optimal rate of CD34+ necessary for hematopoietic reconstitution is unknown with certainty, and a minimum of 2.0 to 3.0×10^6 CD34+ cells/kg is typically recommended. In this work, all the studies have achieved sufficient levels of CD34+, that was 4.26×10^6/kg (MM) and 4.47×10^6/kg (lymphoma), to perform autografts, and no mobilization failure was reported.

Conservation and Cell Viability

Many studies have shown the possibility to save the PBPCs at +4 °C in a refrigerator, for several days, with final viability of over 80% that allows the achievement of autografts (Ahmed et al. 1991; Sierra et al. 1993; Preti et al. 1994; Hechler et al. 1996). Preti and colleagues showed no difference in terms of cell viability and engraftment rate between their maintenance at +4 °C and cryopreservation of PBPCs (Preti et al. 1994). In addition, cryopreservation requires the use of a

cryoprotectant, dimethyl sulfoxide (DMSO), which is responsible for several side effects (Hoyt et al. 2000; Windrum and Morris 2003) so that it necessitates a PBPCs washing before their use. On the other hand, it allows multiple transplantations if and when needed. In all published articles, the conservation at +4 °C allowed the achievement of autografts with a satisfactory rate of the viability of PBPCs despite retention periods up to 3 days, as previously reported in the studies of Cuellar-Ambrosi et al. and Bekadja et al. wherein the storage time was up to 6 days (Cuellar-Ambrosi et al. 2004; Bekadja et al. 2021).

Therapeutic Intensification

Using high-dose chemotherapy (HD), therapeutic intensification is the most crucial part of the autograft procedure, as it has a direct antitumor effect. Since the 1990s, the high-dose chemotherapy of Melphalan at 200 mg/m^2 on D-1, followed by the autologous transplant of PBPCs, is considered the standard first-line treatment of MM for eligible patients to this procedure. So, the schedule consisting of administration of the HD chemotherapy (Melphalan) on D-1 enables the conservation of PBPCs at +4 °C for only 24–48 h, with the viability of CD34+ cells over 95%. This is consistent with an autologous transplant of non-cryopreserved PBPCs. In lymphomas, the situation is very different; the intensification protocols used are shown in Table 2. These protocols, such as CBV (Lobo et al. 1991), BEAM (Smith and Sweetenham 1995), or EAM, include administration periods ranging from 3 to 6 days, which need a collection of $\geq 3 \times 10^6$ CD34+/kg for varying viability of 75% to 85% (Bekadja et al. 2018). The conservation of the PBPCs up to 7 or 8 days is then feasible, but needs to obtain a number of CD34+ cells $\geq 3 \times 10^6$/kg at the time of the reinfusion of the PBPCs. Hence, the significant difficulty is in the mobilization of cells, especially in patients who received multiple lines of therapy. In the consolidation phase, especially in the mantle cell lymphoma or in the diffuse large B cell lymphoma (DLBCL) (Fitoussi et al. 2011; Visani et al. 2012), the probability of obtaining CD34+ cells $\geq 3 \times 10^6$/kg is very high, especially in view of the low number of chemotherapy lines, and non-cryopreserved PBPCs can be used easily. Improving the autograft results in lymphomas will undoubtedly reduce the number of chemotherapy cycles, by early assessment of the PET scan response, and by the availability of new intensification protocols, more myeloablative, as consolidation phase after the first-line induction. These regimens will allow the use of non-cryopreserved PBPCs in view of the high possibilities of their mobilization.

Donor Cell Engraftment

Engraftment was evidenced by the rate of the ANC and platelets count. The aplastic phase was managed either with or without growth factors in case of profound neutropenia. Globally the median length of the ANC and platelets rate was similar

to that of autografts using cryopreserved PBPCs (Lanza et al. 2013) both in the MM (Kristinsson et al. 2014) and lymphoma. Nevertheless in the lymphoma, duration was a little longer due to the number of previous chemotherapy and the refractory nature of lymphoma (Stiff et al. 2013; Rancea et al. 2013; Cook et al. 2014). Overall, these hospitalization numbers show less than 21 days in the MM and 25 days in the lymphoma, which classifies the non-cryopreserved autologous transplant in the favorable group according to Lanza et al. (2013). No engraftment failure or complications related to the infusion of PBPCs were mentioned. In addition, among the 1541 autografts, 4.39% of patients died due to the procedure and procedural complications, particularly in the autologous transplants in lymphoma. This rate of TRM is comparable to that found in the literature, demonstrating the safety of non-cryopreserved autologous PBPCs. Thus, the technique of autologous with non-cryopreserved PBPCs is a simple, reliable, and clinically feasible method. It is also safe, effective, and less costly. Only very few clinical facilities and groups in the developing countries use this procedure, whereas care is essential, especially in hematologic malignancies.

Some authors question the results obtained with unfrozen cells. Their arguments are the difficulties of comparability outside of randomized clinical studies because the characteristics of the diseases and the intensification protocols are not the same (Gale and Ruiz-Arguelles 2018). Although this argument is relevant, it should be kept in mind that carrying out such a study is very difficult and challenging, and for multiple myeloma, the comparability is valid and accurate because the intensification regimen is the same, Melphalan at 200 mg/m^2. The comparability results obtained in multiple myeloma can thus be applied in lymphomas despite the different consolidation regimens. On the other hand, many centers have performed autografts with unfrozen cells and have reported less toxicity and identical efficiency to those of frozen cells (Bekadja and Bouhass 2015).

Currently, more than 1500 autografts have been performed with unfrozen cells, and many studies have retrospectively compared autografts with frozen and unfrozen stem cells. All the results show the non-inferiority of unfrozen stem cells. The indication for autografting of unfrozen cells in multiple myeloma is the most important because:

1. The duration of unfrozen stem cells is less than 48 h.
2. A second autograft is very rare, less than 1% in case of relapse.
3. There are now new drugs to treat relapses effectively.
4. The storage of grafts can pose many problems regarding storage capacities and the need to destroy the grafts after the validity period expiration.

On the other hand, in lymphomas, unfrozen stem cells can only be used as the first consolidation, given the high probability of stem cell mobilization. The length of the conservation is 6 days on average. In the forms of relapse, the possibility of mobilization is less, and it is necessary to use Plerixafor (Mozobil) for mobilization of stem cells (Fricker 2013) to have very high grafts, which can be preserved for more than 6 days. Under these particular conditions, freezing at −180 °C can be

used, which requires less expensive equipment and no liquid nitrogen. On the other hand, in Hodgkin's lymphoma and non-Hodgkin's lymphoma, the probability of relapse is very low and there are also many drugs that are effective against relapses.

Conclusion and Future Perspective

These analyses of the published studies included in this chapter show that performing auto-HSCT without cryopreservation is possible. Although the toxicity and directly related side effects of DMSO infusion are negligible in the study population of CRYO patients, non-cryopreserved products were associated with a lower incidence of severe mucositis and febrile neutropenia. Hence, a significantly shorter hospital stay is required without sacrificing the overall response rate and survival. These results are of utmost significance because they favor better treatment tolerance and, by being cost-effective, they give obvious relief to the increasingly overloaded and already overwhelmed public health care systems.

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A Multilevel Approach to the Causes of Genetic Instability in Stem Cells

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Elio A. Prieto Gonzalez

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E. A. Prieto Gonzalez (✉)
Centre for Advanced Studies in Humanities and Health Sciences, Interamerican Open University, Buenos Aires, Argentina

ISALUD University, Nutrition Career, Buenos Aires, Argentina
e-mail: Elio.Prieto@UAI.edu.ar

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Abstract

This chapter addresses the genetic instability in stem cells, a central feature that is an important determinant for the safety and effectiveness of cell-based therapies. First, DNA damage response mechanism and the gene network that regulates the DNA integrity homeostasis are revisited, focusing on relationship between genetic integrity and stemness maintenance. Also, several factors have been included that influence genetic instability in vivo, i.e., ROS generation and inflammation, and in vitro regarding cell isolation, culture conditions, i.e., oxygen levels and the use of feeder layers and different carriers, splitting procedures, passage number, etc. Telomere shortening, replicative senescence aneuploidy, and the senescence-associated secretory phenotype are different processes involved in deterioration of stem cells abilities for homing, reparability, and paracrine modulation. Moreover, less effective DNA repair upon senescence increases the propensity of developing tumors for stem cells that is one the main concerns related to genetic instability in stem cells. Nuclear organization and their determinants linked to chromosome aberrations and aneuploidy in stem cells and those epigenetic changes affecting stability are addressed. Differences between adipose, embryonic, and induced pluripotent stem cells are analyzed regarding to their ontogeny, changes in culture, and variation in proliferative capacity and stemness. The effect of reprogramming methods in genetic instability and variation in mutagenicity according to genes utilized for pluripotency induction, i.e., Yamanaka's factors and others, is discussed. Donor-related factors, i.e., age, smoking, and alcohol consumption, comorbidities, i.e., obesity, insulin resistance, diabetes, are mentioned for wide evaluation of genetic instability. The next years must witness consensus protocols that will contribute to control the effects of factors that alter stem cell genetic stability to increase the security and applicability of cell-based therapy.

Keywords

Genetic · Instability · Reprogramming · Stem cells · Stemness · Telomere

Abbreviations

ASC	Adipose stem cells
AT	Adipose tissue
ATM	Ataxia telangiectasia mutated
ATR	ATM- and Rad3-related serine/threonine kinase
BATF	Basic leucine zipper transcription factor – ATF-like
BER	Base excision repair
BID	BH3 interacting domain death agonist
BM-hMSC	Bone marrow human mesenchymal stem cell
BRCA1	BReast CAncer gene 1
CASP	Caspase
Cdc25a	Cycle regulator phosphatase

CDK	Cyclin-dependent kinase p16Ink4a
CENP-A	Centromere histone H3 variant
Cernunnos/XLF	Nonhomologous end-joining factor 1
CHK1/2	Checkpoint kinase 1 and 2
c-MYC	Transcription factor, proto-oncogene
CNVs	Copy number variations
CT	Chromosome territories
DBC1	Deleted in bladder cancer protein 1
DDR	DNA damage response
DDT	DNA damage tolerance
DNA-PKcs	DNA-dependent protein kinase
DPPA3	Developmental pluripotency-associated protein-3
DSBs	Double-stranded breaks
EPC	Endothelial progenitor cells
ESCs	Embryonic stem cells
Exo1	Exonuclease 1
FISH	Fluorescent in situ hybridization
FOXO	Forkhead box O1 transcription factor
hADSC	Human adipose-derived stem cells
hESCs	Human ESCs
HIF-1α	Hypoxia-inducible factor-1α
hiPSCs	Human induced pluripotent stem cells
hPSCs	Human pluripotent stem cells
HR	Homologous recombination
HSC	Hematopoietic stem cells
hTR	Noncoding human telomerase RNA
ICLR	Interstrand cross-link repair
IDH2	Isocitrate dehydrogenase-2
iPSCs	Induced pluripotent stem cells
Klf4	Krüppel-like factor 4
LIN28A	Lin-28 homolog A, RNA-binding protein
MAPK p38	Mitogen-activated protein kinase 38
MDC1	Mediator of DNA damage checkpoint 1
mESC	Mouse embryonic stem cells
MMR	Mismatch repair
MN/CB	Micronucleus with cytochalasin B
MNR	Trimeric complex (Mre11, Rad50, and Nbs1)
MRE11	Homolog double-strand break repair nuclease
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
MUSE	Multilineage-differentiating stress-enduring
NANOG	Nanog homeobox transcription factor
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NF-κB	Nuclear factor κB

NHEJ	Nonhomologous end joining
NPM2	Nucleoplasmin-2
NSC	Neural stem cells
Oct-4	Octamer-binding transcription factor 4, POU5F1
p21/WAF1	Cyclin-dependent kinase inhibitor 1A
p53	p53 tumor suppressor gene
PARP1	Poly-ADP ribose polymerase 1
PBMC	Peripheral blood mononuclear cells
Pi3KK	Phosphatidylinositol 3-kinase-related kinase
PPR	Post-replication repair
PSCs	Pluripotent stem cells
RAD17	Checkpoint clamp loader component
RAD18	RAD18 E3 ubiquitin protein ligase
RAD50	Double-strand break repair protein
RAD9	Cell cycle checkpoint control gene RAD9A
Rb	Rb tumor suppressor gene
ROS	Reactive oxygen species
RS	Replicative stress
RT	Replication-timing
SASP	Senescence-associated secretory phenotype
SNP	Single nucleotide polymorphism
SOX-2	Transcription factor SRY (sex determining region Y)-box
TAD	Topological associated domains
TCR	Transcription coupled repair
TLS	Trans-lesion synthesis
TRA-1-60	Pluripotency stem cell marker
USSCs	Human unrestricted somatic stem cells
XPA XPA	DNA damage recognition and repair factor
γH2AX	Histone H2AX

Introduction

In stem cells, genomic instability is crucial and determines the suitability for a proper and safe use in stem cell-based therapy. One of the interpretations of genomic instability is the accumulation of changes that modify genome structure, a situation that is manifested as the sustained increase in translocations, deletions, or duplications that may result in chromosome number variation, but also when is undetectable through karyotype analysis and occurs at molecular level without evident ploidy alterations, referred as the copy number variations (CNVs) (Shen 2011).

Genome stability relies on cellular processes that controls DNA metabolism, i.e., replication, transcription, damage repair, and chromatin remodeling. Those process that are implicit in DNA functioning must be tightly coordinated through checkpoints controlling passages between cell cycle stages, allowing progression and detention when is needed (Hartwell 1992). The cellular checkpoints convey signals

that may trigger DNA repair so as to assure the fidelity of genetic information transmission between cells generations. Along cell cycle, there are different checkpoints that may slow its progression to facilitate damage repair, e.g., M phase checkpoint that guarantees the completeness of DNA replication before mitosis begins. Indeed homeostasis in cells and tissues are preserved via apoptosis induction if DNA damage is too high to be fixed by DNA repair mechanism (Lo Furno et al. 2016; Toledo 2020; Giacosa et al. 2021).

DNA Damage Response in Stem Cells

DNA repair mechanisms are intended to restore the integrity of genetic message, eliminating those changes in DNA molecules that could affect the fidelity of genetic information. These include nucleotide excision repair (NER), base excision repair short and long (BER), mismatch repair (MMR), and transcription coupled repair (TCR). Also, the error free homologous recombination (HR) and those error-prone mechanisms like nonhomologous end-joining repair, interstrand cross-link repair (ICLR), and post-replication repair (PPR). Malfunction of any of these mechanisms may increase genetic instability via generation of chromosomal or sub-chromosomal aberrations or indirectly through provoking mutation affecting repair checkpoints genes or other genes involved in DDR that may affect genomic stability and result in a mutated phenotype. In fact there are many reports on the overexpression of genes responsible for most of the DDR mechanism in stem progenitor cells compared with those active in adult mature cells. DDR in stem cells have been recently reviewed (Mani et al. 2020).

There are many gene families that are involved in DNA damage detection, signaling, and processing; one of them is the phosphatidylinositol 3-kinase-related kinase (Pi3KK) which includes ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PKcs), and ATM- and Rad3-related serine/threonine kinase (ATR), and those enzymes are implicated in the maintenance of genomic instability in stem cells. In mesenchymal stem cells (MSCs), there are evidences of linking ATM decline with progressive lowering of the ability to respond to replicative errors as well to genotoxic damage that arises during long-term expansion in culture (Shiloh and Ziv 2013; Beckta et al. 2017). ATM plays a principal role in repairing the highly lethal double-strand breaks (DSB). ATM responds mainly to DSB and to changes in chromatin DSB, while ATR become active after nucleotide damages, impairment in replication-fork progression and also to DSB. DNA-PKcs is linked to NHEJ. CHK1/2 favors DNA repair by reducing the cell cycle progression through modification in the state of master cell cycle regulators like CDC25 proteins (Lo Furno et al. 2016).

Pi3kk triggers a phosphorylation cascade that modulates the activation of downstream genes in the repair circuit, i.e., p53, BRCA1, and CHK1. Protein products of that genes are assembled in the reparosome at DNA damage sites and provokes several relevant actions beside DNA repair, i.e., promoting the blockade of DNA replication and mitosis until DNA damage is fixed; however, in certain

circumstances, the apoptotic response may also be induced. Phosphorylation of histone H2AX (γH2AX) that is a reliable signal of genetic instability is a consequence of ATR action, that is triggered by single-strand regions that appears if replication fork is delayed or stopped at damaged sites in DNA. ATR is also activated when double-strand breaks are present (Lo Furno et al. 2016).

ATM is combined to trimeric complex MNR (Mre11, Rad50, and Nbs1) that is gathered at DSB sites, keeping the extremes of the gaps to be repaired. ATM is activated through shifting to an active monomer that begins to acts as a kinase over the substrates P53, BRCA1, CHK2, RAD17, RAD9, and NBS1 in order to build up repair foci. ATM is able to phosphorylate MDC1, Rad18, p53-binding protein 1 (53BP1), and BRCA1 to trigger DBSs repair of the two main mechanism involved in this branch of DDR, homologous recombination (HR) or nonhomologous end-joining repair (NHEJ) (Lukas et al. 2011; Nair et al. 2017). DNA damage response mechanisms are crucial to avoid the deleterious effects of DSB that are repaired mainly through NHEJ mechanism that is related to the ATM pathway.

An example of the significance of ATM for DNA damage repair response is seen in MSCs from FVB/N mice strain, which expressed a significant increase in micronucleus with cytochalasin B (MN/CB) frequency, both spontaneous and related to gamma irradiation, and also in DSB. Frequency of both lesions of MN/CB and DSB showed an additive effect of aging and genotoxic stress. Correspondingly these cells diminished their ability to detect DNA damage, as expressed in less γH2AX/53BP1 DNA foci formation, less DNA repair, and increased residual DNA breakage when cultured over 8 weeks. In the first week of culture, a strong response of ATM was present, but 8 weeks later, ATM impairment resulted in reduced DDR (Hladik et al. 2019).

ATM importance for genetic instability in stem cells can't be underestimated. The p53/ATM network is involved in DNA damage recognition and the triggering of DDR but also in the initiation of apoptosis. Notably, induced pluripotent stem cells (iPSCs), that shows a reduced less ATM phosphorylation activity, exhibits an increased apoptotic response when treated with low level radiation. There are evidences that cells affected by certain mutations in p53 develops an increase in interchromosomal translocations and defective G (2)/M checkpoint. Those p53 gain of function mutations promote the association of p53 to Mre-11 nuclease therefore affecting the assembly of MNR complex (Mre11–Rad50–NBS1), and this malfunction of MNR impairs its binding to DNA DSB that results in a deficient triggering of ATM function (Song et al. 2007). There is a report on the abnormal karyotype in knockout ATM tail-tip fibroblasts that albeit exhibited a lower reprogramming efficiency, their descendant iIPS conserved their pluripotency for 20 passages. A result that emphasizes the main role of ATM in stem cells genetic stability, but also is a warning for the possibility therapeutic use of genetic unstable cells that in spite of that risky conditions may be able to proliferate and differentiate, eventually in a tumor growth (Kinoshita et al. 2011).

There are reports connecting low activity in ATM with reduction in iPSCs differentiation as well as increased genomic instability (Nagaria et al. 2016). Efficiency in reprogramming cells to iPSCs is reduced in cells where ATM pathway is

impaired and is associated with more genomic variation. When was evaluated the effect on genomic variation over the frequency of CNV in the boundaries of ATM gene, those ATM-CNV bearing iPSCs showed a higher susceptibility to genome instability than wild type cells. The greater effect of impaired DDR resultant from a higher frequency of CNV affecting the gene provoking ATM-deficiency was evident at earlier stages after retrovirus-based reprogramming (Lu et al. 2016).

There is a group of effectors that participate in the choice between proliferation and differentiation in stem cells. These processes are strictly regulated so as to favor the right number of and the kind of pluripotent cells in each moment during embryogenesis and organogenesis. The coordinated generation and the proper decision in each cell population is also necessary for the repair and regeneration of damaged tissues, or in response to renovation needs in the adult individual. Among those proteins that are involved in the control of those orchestrated decisions is geminin that is a target for the cyclosome, or anaphase-promoting complex. Geminin acts as an inhibitor of pre-replication through binding to Cdt1 pre-replicative complex and avoids alterations in replication timing. Geminin expression is greater in late S phase and during G2, and this factor is also a key element in the neurogenesis (Kushwaha et al. 2016). Geminin interacts with members of the suppressive polycomb protein family as well as with the trithorax group of proteins that regulates neuronal development. The tuning in these network allows geminin to participate in the regulation of Hox genes among many others that controls the differentiation routes in progenitor cells (Kingston and Tamkun 2014; Lau and So 2015).

Geminin prevents pre-replication of DNA that may lead to re-replication and genomic instability. However, it seems not to be a universal characteristic, as is evident in the fact that abrogation of geminin in a murine model for brain and hematopoietic development did not increase genomic instability nor the frequency of DNA damage in this model. The complexity of its actions does not seem to be restricted to genome protection, and even this property is controversial because geminin overexpression is present in cancer cells, a "Janus" behavior that is exemplified in the geminin-induced upregulation of 24 genes involved in transition from epithelial to triple negative breast cancer metastasis, homing and stemness in cancer stem cells. Geminin is an example of the series of factors that participate in genomic stability maintenance and could be considered as next target for genetic stability interventions (Sami et al. 2021; Montanari et al. 2006).

Factors That Influence DNA Stability in Stem Cells

One of the aspects that must be addressed when considering the requirements for stem cell therapeutic applications is the genomic instability, because it is a condition that could hamper the outcome of stem cell transplantation. Microenvironment, where stem cells are introduced in order to be expanded, poses a threat to cells stability. The delicate homeostatic balance in the transplanted cell is affected by several factors like oxygen level, pH, and inflammation in donor tissues and ROS (reactive oxygen species) levels in vivo and ex vivo, among several ones

(Aerts-Kaya 2020). Superoxide anion as well as hydrogen peroxide are generated not only in donor environment but also during handling; hydrogen peroxide is not a ROS but can be easily converted in that, through Fenton or Haber Weiss reactions, where transition metals participates. Hydrogen peroxide is also involved in intracellular signaling as well in pathogen destruction when is liberated by immune cells like neutrophils. ROS are well-known DNA damaging agents that may lead to genomic instability (Barzilai and Yamamoto 2004; Seddon et al. 2021).

Among the factors that increased mutation frequency in hESCs (human ESCs), one that cannot be excluded is the composition of culture medium, the origin of feeder layer, the passaging method utilized, the freeze thawing procedure, and oxygen level, that is known to generate more instability at normoxia (21%) than at hypoxia (2%). The plate or flasks where cells are cultured influence stem cell fate, and it have been reported that expansion of ESCs in microcarriers for several months conserves pluripotency besides their cytogenetic stability and normal karyotype (Oliveira et al. 2014).

Oxidative Stress

DNA can be damaged by oxidative stress generating a large array of base modifications being the commonest 8-oxoguanine, a lesion that is not only highly mutagenic but also accumulates with age. This lesion triggers base excision repair (BER) a mechanism that removes the damaged base and produces a single-strand break in the DNA before filling it with a new non-affected base. Strand breaks in one or both strands induces poly-ADP ribose polymerase 1 (PARP1), an enzyme that is more active with age, and leads to a greater frequency of DNA breaks in older subjects (Petr et al. 2020).

Hematopoietic stem cells (HSCs) utilizes a preventive strategy so as to avoid ROS-provoked DNA damage based on the maintenance of a hypoxic state (Goto et al. 2014; Testa et al. 2016). This implies a substitution of cell respiration in mitochondria by glycolysis in order to produce ATP, and shifting again toward mitochondrial respiration when cells reenter cell cycle. This contraption works as an antioxidant defense mechanism, because respiration ROS generation is lowered and consequently DNA oxidative damage is reduced (Takubo et al. 2013). Cells during culture experiences high metabolic exigence that increases as culture time does and the cells experienced a greater number of passages. High metabolic rate is a cause of leakage in the electronic transport chain that leads to ROS generation and genetic damage. The margin for ROS levels in cells that undergoes reprogramming is narrow. Low levels affect reprogramming while high impairs differentiation. That poses an strict dependence of reprogramming success on the correspondence of oxygen level in culture with the cell type, its age and the specific characteristic of culture conditions (Henry et al. 2019; Zhou et al. 2016; Rönn et al. 2017). The recent demonstration of the role of NADPH oxidase, a master factor in oxidative balance, in the fate of stem cells, remarks the importance of the maintenance of oxidative homeostasis over stemness during ex vivo cells expansion (Maraldi et al. 2021).

When DNA mutations favors an increase in ROS generation, the self-renewal capacity of HSCs is hampered. ROS generation is augmented when ATM, a checkpoint for DNA damage, is abrogated. This is a curious relationship between a component of DDR, ROS generation, and cell cycle progression in HSCs. The higher ROS production reduces self-renewal capacity through activation of cyclin-dependent kinase (CDK) p16Ink4a and the tumor suppressor gene Rb, but in addition, interrupts quiescent state by inducing p38 kinase (Ito et al. 2004). This effect have also been observed in neural stem cells (NSCs) (Kim and Wong 2009); it is an expression of the importance of ATM pathway in DNA damage response against different stressors and the interconnection between a DNA damaging responsive kinase like ATM and induction of oxidative stress (Kitagawa and Kastan 2005).

Another link with DNA function is that of FOXO, a transcription factor that is induced in response to oxidative stress, stimulates Rb and p53 activation that reduces quiescence and self-renewal in these cells. There is a relative antagonism between quiescence and ROS generation that favors reentering cell cycle through reactivation cyclin-dependent kinase. FOXO p53 and Rb are necessary to limit oxidative stress DNA damage and genome instability (Bigarella et al. 2014; Burkhalter et al. 2015). The main HSCs functions, self-renewal and differentiation, are controlled by intrinsic mechanisms, transcriptional and epigenetic regulator hormones, and cytokines but also by the close bone marrow microenvironment. HSCs are commonly assumed to reside within the hypoxic bone marrow microenvironment. It must be remarked that there are striking differences between metabolic pathways that are actives in HSCs and adult differentiated cells. In the former, responses to hypoxia are derived by hypoxia-inducible factors, dimeric Hif-1 and Hif-2, which are oxygen-regulated alpha (Hif-1a and Hif-2a) and beta subunits (such as Hif-1b or Arnt1) that modulate gene expression during adaptation to hypoxia. There is genetic evidence supporting the assumption that deficiency in Hif-1a in HSCs lowered the expression of pyruvate dehydrogenase kinase (Pdk2) that is able to enhance the "against ROS-protective glycolysis." Hypoxia is recognized as an important factor that regulates stem cell quiescence status and metabolic shift toward energetic metabolism based in glycolysis and pentose phosphate metabolism, instead of oxidative phosphorylation in order to lower ROS generation (Forsyth et al. 2008).

Low oxygen level conditioned proliferation and differentiation in ESC, cells that possess an efficient antioxidant mechanism that counteract the effect of variation of oxygen tension. It is noteworthy that when ROS become higher, the pluripotency genes OCT4, Nanog, Tra 1-60, and Sox2 lower its expression as well as differentiation is triggered. Oxygen conditions must be finely syntonized and 2% that is below physiological conditions is recommended as secure for genetic stability and stemness (Forsyth et al. 2008).

When cells become differentiated, mitochondrial respiration is resumed, a change that prompts an increase in oxidative stress and higher probabilities for DNA damage and mutations. Thus transition from quiescence and glycolysis toward respiration and ROS generation contributes to mutagenic oxidative lesions in HSCs that foster stem cell genetic instability. As an example, in acute myeloid leukemia cells, mutations in the gene of mitochondrial enzyme isocitrate

dehydrogenase-2 (IDH2) function as an oncogenic driver (Gezer et al. 2014; Testa et al. 2016). Hypoxia should be considered as a way to limit ROS damage, but also as a condition for development of bad prognosis characteristics in tumor cells. That is in agreement with that literature pointing to activation of the hypoxic response in tumors, mediated through upregulation of hypoxia-inducible transcription factors (HIFs), that are linked to genomic instability, and increased metastatic potential, and tumor stem cell characteristics among other worse phenotypic features (Böğürcü et al. 2018; Tong et al. 2018).

Senescence as a Factor Inducing Stem Cells Genomic Instability

Senescence is a stable cellular program that induces growth arrest and result in a distinguishable phenotype, involving chromatin remodeling, metabolism reprogramming, a characteristic inflammatory secretome, and high autophagy. Activation of p16INK4a/Rb and p53/p21CIP1 tumor suppressor genes are determinants for the arrest in cell proliferation so as to impede the possibility of gene transmission to next cell generation. Among the triggers for senescence can be mentioned, DNA damage, mitochondrial dysfunction, telomere shortening, pro-carcinogenic aggressions, metabolic stress, and epigenetic changes. Senescence is a process relevant to tissue homeostasis, cancer, and aging. There can be two kinds of senescence: that which is caused by external causes that is acute and the chronic senescence related to DNA damage and replicative stress. That means that senescence is related to genetic instability in dependence of the kind of unrepaired DNA damage that accumulates and eventually may deteriorate the stem cells therapeutic properties that relies upon tissue cell renewal and secretome paracrine actions (Schmitt 2016; McHugh and Gil 2018; Neri and Borzi 2020).

Senescence is related to the action of regulators like mTOR, the serine/threonine kinase that controls metabolism and cell growth according to nutrients, cell energy level, or other sources of stress. mTOR contributes to regulate such processes as nutrient intake, protein and lipid synthesis, autophagy, and the emergence of the senescence-associated secretory phenotype (SASP) that participates in the autocrine and paracrine senescence induction. Senescence is also linked to MAPK p38 activation or induction of p16INK4a, in response to several disturbances already mentioned, like telomere shortening, oxidative stress, oncogene activation, and DNA damage. Another factor that promotes p53 degradation bypassing and senescence are the Sirtuins (SIRT, that will be addressed in the following sections) (McHugh and Gil 2018).

Aging and Genomic Instability in Stem Cells

Accumulation of mutations in the whole genome suggest that homeostatic mechanisms worsens during aging (Garinis et al. 2008; Martincorena et al. 2015), that is considered a reason for the greater amount of IPSCs bearing mutations, some of

those have been found in white blood and skin cells, in genes linked to cancer development (Jaiswal et al. 2014; Genovese et al. 2014). Also in mitochondrial DNA, there have also been shown a mounting number of mutations in aged cells. In iPSCs bearing mitochondrial DNA mutations, the organelle is dysfunctional and provokes further metabolic disturbances (Kang et al. 2016; Wang et al. 2011), and those alterations in the mitochondria propelled further DNA damage in nuclear DNA.

Another evidence of the deleterious effect of aging on IPSCs therapeutic efficacy comes from IPSCs obtained from elder mice. These cells with age-associated greater mutation level exhibited less proliferation and reprogramming capacity (Wang et al. 2011). Increase in stem cells age and mutation burden results in deterioration of damage cell responses that affects stress tolerance, tissue and organs functions, and regeneration capacity, all of them disturbances that could lead to cancer and age-related conditions. Mutations may arise in totally stochastic way, accumulating as cell ages. However, there are evidences of reduced checkpoint activities in older cells that often results in exponential increase in mutation frequency. In that sense, aged iPSCs provides a favorable scenario for genomic instability (Burkhalter et al. 2015). Burkhalter et al. (2015) have reviewed the evidences of mutation path from aging to disease. HSCs exhibits a slower DNA repair that contributes to more prevalent mutations. These HSCs mutations arose randomly and are neutral or passenger until a driver co-mutation may increase the fitness of bearing cells. If a second mutation occurs, it may lead to a greater proportion of cells with stable and transmissible changes in their genetic information (Welch et al. 2012). Sequence of phenotype changes associated to mutations reveals that in the first stage, mutations may increase self-renewal, but in the following stages, another driver mutation may help the HSCs to become a leukemic stem cancer cell. This process has been shown also to develop in male germ cells, favoring clonal expansion and the transmission of mutations (Jan and Majeti 2013; Minussi et al. 2021; Reilly and Doulatov 2021).

The higher mutation frequency and telomere shortening in older HSCs could be related to DNA repair impairment during quiescence (Beerman et al. 2014; Wang et al. 2014). In HSCs, quiescent DNA damage is not repaired, but when cells reenter cell cycle, HR begins to proceed again, however, as is widely known, if DNA damage is not reversed, the pool of genetically dysfunctional cells is cleared through induction of apoptosis or senescence. It has been observed that DDR are negatively affected during aging process. Nevertheless, there are many unknown areas regarding of DNA repair mechanism during physiological aging that are under scrutiny (Ko et al. 2010).

Our knowledge on DNA repair and aging arouse mainly from the study of progeria syndromes DNA damage and cancer. This is an open research avenue that will impact in the study of the aging influence in the genomic stability of stem cells utilized in therapy (Hoeijmakers 2009; Song et al. 2021).

Checkpoints are critical for an adequate stem cells function. An example to whom we have previously referred to is the DNA damage detection network that includes both ATM-Chk2 checkpoint kinase and Ataxia telangiectasia and Rad3 related (ATR). These kinases promotes p53 and Rb activity as a part of the DNA damage

response, whose nature is affected by the damage origin and burden. There are two main results: one is transient, including self-renewal suspension or quiescence, while the other triggers senescence or apoptosis that is a more definitive solution that contribute to remove damaged cells. The amount of stem cell that is required to replenish the tissue/organ that have deleted the injured stem cells depends on the return to quiescence and self-renewal, which need the expression of growth factors. Growth factors induce cell divisions so as to recover stem cell number. Some authors have proposed that removal of damaged cells mediated by p53 must occur in order to guarantee progenitor cell driven renewal, an option that relies on undisturbed naive cells harboring a more preserved genetic endowment (Schoppy et al. 2010; Smith et al. 2010).

If the checkpoints ATR or Chk1 are eliminated, stemness is much compromised and a premature aging affects HSCs, and this trouble can also be observed in cells from bone, skin, and small intestine. Absence of CHk1 and ATR allows a mounting number of DNA lesions that accumulates and trigger cell cycle incapacity to progress and provokes apoptosis (Ruzankina et al. 2007; Desmarais et al. 2012). It is evident that a precise tune of the activity of these checkpoints is indispensable. Absence of their function is deleterious while its activity may result in senescence or apoptosis depending of the magnitude of damage in DNA (Shibata et al. 2010; Lossaint et al. 2011).

Stem cells aging impairs its replicative capacity, and it is notorious that, during aging, several kinds of stem cells, like satellite and HSCs, stay in quiescence that helps in attaining DNA repair (Moehrle et al. 2015). However, desrepression of p16INK4a in muscle satellite cells induces a senescence-like stage that block stem cells to resume the cycle that is exceedingly affected and delayed (Rossi et al. 2007; Sousa-Victor et al. 2014; Flach et al. 2009). Cell cycle delay results from replication stress linked to chromosome gaps or breaks. Replicative stress is related to lowered expression of a helicase M component that is necessary for adequate assembly of replicative complex and affects progression of replication. On the other hand, DNA damage tolerance (DDT) mechanism is activated which allows replicative fork to bypass DNA lesions that otherwise would impede fork progression. It is necessary to remark the importance of this tolerance alternative, because if DDT fails, replication can be stalled, leading to generation of DSB, lesions that are highly toxic and may result in stem cell genomic instability (Pilzecker et al. 2019). Genomic instability as a result of replicative stress is a fact that must be considered when planning cell harvest for expansion and further use in stem cell therapy.

Stem cells suffer injuries in vivo and in vitro, from several sources, like energetic metabolism, and generation of reactive oxygen species, due to inflammation, but also to manipulation, expansion over different matrices, shearing forces among many disturbances that may affect DNA integrity, not only at the beginning of their ex vivo living but alongside their life span in culture. The status of DDR machinery in stem cells and the resulting DNA stress experienced by those cells intended for therapeutic uses is a multivariable, almost random sequence. A situation that makes mandatory not only the standardized for sample selection and cell handling but also for the genomic evaluation previous to use.

Telomere Damage and Genetic Instability in Stem Cells

Telomeres, the chromosome ends, that are abundant in highly repetitive sequences, exert an influence on the whole cell genomic stability. Shortening of telomeres is one of the main features linked to aging (Hastie et al. 1990; Jiang et al. 2007; Smith et al. 2020; Grill and Nandakumar 2020). Moreover, accumulation of DNA damage in telomeres can induce premature aging (Uppuluri et al. 2021). In a progeria condition like Werner syndrome, a helicase dysfunction that favors telomere shortening is related to premature aging. Telomeres extremes are cut of at a rate of 50–100 nucleotides per cell cycle. Those ends are covered by nucleoproteins named shelterin, in order to protect chromosomes terminus from uncontrolled degradation (Henry et al. 2019; Engin and Engin 2021).

There are different shelterins: TRF1, TRF2, RAP1, POT1, TIN1, and TIN2, bound to DNA and in a complex that is called shelterin/telosome, and if this protein complex doesn't work properly, several abnormalities appears in DNA (Liu et al. 2004; Engin and Engin 2021) like fragile sites and replication defects that leads to chromosome fusions and anaphase bridges (Wieczór et al. 2014). Shelterin actively participate in the control of telomere replication and chromosome final shielding. Additionally, DNA damage response after shelterin disruption is also evidence that inadequacy in their function causes genomic instability (Smith et al. 2020; Kabaha and Tzfati 2021). These proteins are affected by oxidative stress, and there are reports on the impairment of shelterin and its association with telomere DNA if it is damaged by oxidation (Opresko et al. 2005). Telomeric oxidative damage has also been implicated in genomic instability that have been linked to protein kinase (p38)-p16(Ink4a) (p16) that participates in ROS-induced HSCs senescence (Shao et al. 2011).

HSCs show active DNA repair pathways to maintain their stemness. Exonuclease 1 (Exo1) participates in HR, that fails to proceed if this enzyme is inactivated. In quiescent HCSs from mice, HR is not necessary for cell maintenance, relying on NHEJ repair instead. However, after HCSs reenter in cell cycling, the HR dependence from Exo-1 activity, for DNA repair and preservation of HCS, becomes critical (Desai et al. 2014; de Lange 2018). There are reports on exonuclease 1 (Exo1) activity that after telomere shortening as component of telomeric DNA damage response, its failure results in chromosome fusion and checkpoint induction, and this fusion causes expansion of DNA damage, chromosome instability, and probably stem cell senescence (Zhang et al. 2014).

Telomerase an enzyme that is present in most of eukaryotes, shows a typical retroviral reverse transcriptase-like protein core and the noncoding human telomerase RNA (hTR). This enzyme limits telomere shortage in stem cells by synthetizing telomere DNA, which protects DNA from critical shortening until number of cell divisions is completed. However, when telomerase is less expressed, it is unable to prevent shortening and affects cell functions like self-renewal in HSCs and IPC (Flores and Blasco 2009; Roake and Artandi 2020). In human cells, telomere shortening reaches critical length that are sensed as DNA damage, a situation that

triggers the cell machinery that copes with DNA lesions and consequently activates p53/p21-related checkpoints. This results in the interruption of cell cycle progression, triggering telomeres senescence, a response to DNA damage checkpoint activation after DNA damage within telomeres (Liu et al. 2019).

Cycle arrest due to DNA damage is known as replicative senescence. Cells undergoing replicative senescence experiments morphological and biochemical changes, such as enlargement or flattening, and a higher expression p21(WAF1) and/or p16(INK4A). Checkpoints like those based in p53 and Rb are responsive to telomere shortening (Sperka et al. 2012; Morsczeck et al. 2019), p53 response may interrupt cell cycle through kinase inhibitor p21 or may trigger apoptosis via induction of Puma factor, so as to eliminate those cells bearing affected telomeres from the stem cell pool that is needed for tissue renovation (Sperka et al. 2011). On the contrary, in spite of that long telomeres can also accumulate damage, their effects are less noxious for stem cells, due to protection afforded by sheltering that avoids checkpoint induction nor triggering of DDR (Fumagalli et al. 2012; Sławińska and Krupa 2021).

Human ESCs with shorted telomeres shows a reduced pluripotency (Huang et al. 2011). Telomere length conservation is necessary for stemness, unlimited self-renewal, and chromosomal stability of stem cells. Telomerase activity is necessary but not the only mechanism to guarantee telomere length. Epigenetic changes and recombination are also relevant during reprogramming and attainment of pluripotency in ESCs and IPSCs (Liu 2017; Wang et al. 2012a, b). It is noteworthy that long telomeres as well as high levels of TRF1, a shelterin, can be considered as markers of stemness. On the other side, it is important to note that higher levels of telomerase, in spite of boosting self-renewal and proliferation, also induce a greater resistance to apoptosis and have been linked to less differentiation capacity (Flores and Blasco 2009).

Senescence is a process characterized by proliferative arrest. This contingence could benefit the cell trough tumor suppression or may contribute to tissue repair. As was stated previously DNA damage can initiate senescence; eventually cells increase protein secretion and many of tis proteins are cytokines; the resulting phenotype is termed senescence-associated secretory phenotype (SASP). SASP reflects an outstanding paracrine effects that may induce senescence in other cells. This evidences that senescent cells may spread senescence in their surrounding milieu (Sławińska and Krupa 2021).

Induction of differentiation consecutive to DNA damage is a mechanism that have been demonstrated in several cells like HSC when exposed to aging-associated genotoxic stress. An example refers to induction of basic leucine zipper transcription factor, ATF-like (BATF) that promotes lymphoid differentiation in cells exposed to genotoxic stress (Wang et al. 2012a, b). It is as an alternative checkpoint that allows leading with DNA damage in stem cells, like occurs in melanocyte stem cells in the hair follicle, these cells after DNA damage related to ionizing radiation differentiates to melanocytes, a process that may be further enhanced if ATM is deleted (Inomata et al. 2009).

Aneuploidy as an Expression of Genome Instability

Among the causes of aneuploidy in stem cells that compromise their therapeutic usefulness, an important one is the disturbances in telomerase function. In hESCs, the enzyme is constitutively active in order to maintain telomere length, and in the case of iPSCs, telomerase activity is restarted after reprogramming (Marión et al. 2009). Telomere fusion poses a great problem in anaphase because those connected chromosomes may generate anaphase bridges that are impeded to migrate toward opposite cell poles under the mitotic spindle tubules action. Cells remain linked through the bridge that causes aneuploidy because of the illicit gain of a chromosome in one cell whereas the other suffers a loss. Telomeric shortening is a common cause for anaphase bridges that can be broken generating cycles of breakage–fusion, a situation that exhibits proneness to genomic instability and aneuploidy (Tusell et al. 2010; Srinivas et al. 2020; Henry et al. 2019).

There are several and sustained evidences that telomere damage may trigger genomic instability expressed as an outburst of chromosomal aberrations as well at the sequence variant level, when those chromosome ends are disturbed, telomeres experience damage and attrition (Henry et al. 2019; Kim et al. 2021).

Stem cells centromeres does not function with the same efficiency as those in mature cells, and this characteristic is probably a cause of predisposition to aneuploidy in those cells. Chromosomal aberrations are frequently found in IPSCs in culture, and among the probable causes for those abnormalities are the composition of centromere and kinetochore in ESC and IPSCs. Kinetochore is the critical spot for chromosome attachment that relies on CENP-A, the centromere histone H3 variant, or nucleosome defined chromatin. There are reports on reduced amounts of this centromere component in stem cells that could hamper chromosomal-spindle attachment. During reprogramming, CENP-A is reduced at the beginning of dedifferentiation events leading to IPSCs (Milagre et al. 2020). This acts as an epigenetic regulator of stemness, which in turn favors aneuploidy in pluripotent stem cells. Overexpression of that critical centromeric component occurs in cells with functional p53 that are able to drive cell reprogramming toward senescence, tumorigenesis, or to new centromeres linked to aberrant chromosomes (Ling et al. 2020; Jeffery et al. 2021), which is interpreted as a demonstration that a precise tuning of centromere components exerts a decisive influence in the reprogramming results.

Influence of Genomic Arrangement in the Nucleus on Aneuploidy

In the interphase nuclei of differentiated cells, there is an organized distribution of chromosomes in areas termed chromosome territories (CT). Alterations in the intranuclear organization may impact the maintenance of euploidy. Inside human cell nucleus, there is a strict array of CT with predominant gene-rich chromosomal regions placed towards the inner part of nuclei while the gene-poor regions of chromosomes are placed at the nuclear periphery. In spite of some variations, there

is a less organized intranuclear terrain in the embryonic versus mature developed cells, and the rule based in chromosomal gene enrichment is not always followed. An example of the previous assertion are chromosomes 17 and 19 that are abundant in genes and are found in granulocytes near the nuclear center while in ESCs are placed in the periphery (Bártová et al. 2001). However, more recent reports have found differences in centromeres positioning of 12, 15, 17, and 19 between hESC and mature somatic cells. There is a repositioning process that accompanies cell differentiation; impairment in this mechanism can be invoked as an additional source of genomic instability in stem cells (Bártová et al. 2008). This has been reported to influence several features of heterochromatin, as the presence of immature centromere anchorage regions that in embryos and stem cells may result in aneuploidy due to a defective attachment to the mitotic spindle (Biancotti et al. 2010).

The complex machinery for intranuclear topography maintenance also includes nuclear transmembrane proteins that are responsible for gene repositioning, during myogenesis, in order to shift the genes to be repressed to more peripheral positions (Robson et al. 2016; Ahanger et al. 2021). Those peripheral domains are mainly low gene expression regions that contain histone marks of a silenced state (Smith et al. 2021). There is an increasing number of evidences that allows a less restricted nuclear organization in pluripotent cells, hereof increasing the intranuclear plasticity regarding not only to expression and organization but also to genomic instability (Vasudevan et al. 2020; Dhegihan 2021).

Aneuploidy is probably an insult to the intranuclear genome organization because of the additional space occupied by the extra chromosome altering the space relationship with the nuclear envelope or other regions. The correct location of extra chromosomes that have been found in mature somatic cells is not the rule in stem cells, where there are less or not lamins A, (that will be discussed next) with a consequential impact on lamina-associated domains, therefore may alter the intranuclear lodging and hence allowing changes in gene expression (Chovanec et al. 2021).

Aneuploidy-associated shifts in genome organization and subtypes of topologically associated domains (TAD) upon differentiation is under scrutiny (Phillips-Cremins 2014), and is another link in the chain of event connecting the stem cell nuclear environment and aneuploidy proneness and generation of a distressful genomic landscape (Omori et al. 2017).

There are also “counter measures” in stem cells against changes in chromosome number relying on DDR and protective proteins. An example of a mechanism against polyploidy is the protein Survivin that control the spindle assembly, checkpoint and cytokinesis; that protein that is protective against aneuploidy, is highly expressed in ESC and contributes to pluripotency (Wiedemuth et al. 2014). Measurement of this protein has been suggested as control for genomic stability in stem cell intended for therapy (Mull et al. 2014).

Lamins Alterations and Aneuploidy

Lamins are meshing proteins that are placed covering the inner face of the nuclear membrane, and those proteins are important in the conservation of nuclear

morphology and chromosomal location within the nucleoplasm. Lamins B1 and B2 are expressed in stem cells as well as in somatic ones. Lamins are necessary for the conservation of nuclear structure as well as the chromosomal allocation and organization (Gruenbaum et al. 2000). Lamins alterations may lead to miss-segregation of chromosomes during cell division, due to its relationships with mitotic spindle assembly and telomere binding to the inner nuclear membrane protein SUN1. That is why lamins are among the factors that may induce aneuploidy in stem cells in the eventuality of disturbances. There is a link between lamins function impairment, telomere damage, and genomic instability (Gonzalo and Eissenberg 2016; Martins et al. 2020; Dantas et al. 2021).

The absence of lamins A/C has been suggested to contribute to the ESC nuclei plasticity compared to the more rigid state of somatic cell nuclei, with hESC lacking heterochromatin at the nuclear periphery. Lamin A/C and emerin determine nuclear size and shape, and hence these are contributors to gene regulation and differentiation in the first steps of embryo development (Smith et al. 2017) and to global remodeling of the genome organization during lineage commitment (Peric-Hupkes and van Steensel 2010).

Replicative Stress/Replication Timing as Causes of Genome Instability

Replicative stress (RS) is another source of genomic instability in cells undergoing divisions that is present in stem cells. RS main feature is to slow down DNA replication or its complete detention. DNA synthesis can be hampered by several factors as DNA damage, (vb gr) like the inflicted by ROS or when DNA nucleotides or proteins are scarce due to a metabolic impairment RS can also arise (Lo Furno et al. 2016). Among the main causes of RS are the unscheduled restart of cell cycle that provokes interference between DNA replication and transcription but is also frequent during reprogramming into IPSCs. As a consequence of interferences between DNA synthesis and transcription, the replicative fork may lead to under or over DNA replication and also to single- or double-strand DNA breaks. RS is a cause of instability and DDR in stem cells (Lo Furno et al. 2016; Sjakste and Riekstiņa 2021).

In ESCs, there is a susceptibility to RS resulting in defective chromosome condensation and segregation, leading to aneuploidy. ESCs cell cycle contains a higher number of cells in S phase, while G1 and G2 are narrowed. The more prevalence of RS in stem cells may be explained by the fact that during DNA synthesis, chromatin is more accessible to remodeling factors (Becker et al. 2006; Lambert and Carr 2013; Lamm and Kerem 2016). There is another relevant aspect to be considered among the aneuploidy leading events, which include the checkpoints for adequate chromosomal segregation, the spindle assembly, and the decatenation checkpoint. The last checkpoint during mitosis is the p53- and Rb-dependent G1 tetraploid checkpoint. The spindle assembly checkpoint has been reported as less efficient in ESCs, a feature that may lead to extra chromosomes. This spindle checkpoint is also weak, favoring association of unrepaired chromosomes. There

are evidences that points to a lack of spindle assessment checkpoint in stem but not in progenitor cells (Brown et al. 2017; Brown and Geiger 2018). Chromosome decatenation impairment has been found in mouse cells and also in pluripotent human cells. The consequences of bad decatenation are the entanglement of chromosomes that is attributed to the absence of methylation in chromatin silence markers (Meshorer et al. 2006). In hematopoietic stem cells, the levels of Ezh1 histone methyltransferases are progressively reduced during development, and those enzymes are thought to be implicated in methylation of silencing chromatin markers like H3K9 and H3K27 (Hidalgo et al. 2012). The more open heterochromatin in stem cells may be responsible for the greater susceptibility to DNA damage and genome instability observed both in hPSCs and hiIPSCs (Henry et al. 2019).

Another emerging key factor that must be addressed regarding genomic stability is the replication timing (RT), a concerted process that control the organization of a relevant array of nuclear events in eukaryotic cells. RT is triggered at certain point during cell cycle and its development is synchronized with changes in genome organization. RT is connected to processes like 3D genome organization, transcription, epigenetic modulation and mutation, and spatial distribution, and is thought to influence replicative stress and consequently genomic stability (Briu et al. 2021).

Replication process is strictly timed; in the multiple origin replication process, there are regions that begin to be copied at different moments during S phase. This chronometric control of each region for replication results in the epigenetic signature of the cell genome. This process is cell specific and spans large regions of chromatin, where histone modification and compartment changes, which defined the activation of specific replication origins. Those patterns are inherited to next cell generations. However, if there are inaccurate timing as a result of replicative stress, altered gene expression and genetic instability are among the bad results of RT impairment (Donley and Thayer 2013). In other words, replicative stress may cause genetic instability through modifications in RT. In stem cells, the evidence points to a less strict correlation between histones modification and the precise scheduled RT. However, as long as stem cells differentiate, there is a more tight dependence of RT from histone changes as occurs in mature fully developed cells (Dileep et al. 2019).

There is another aspect regarding the effect of increased mutation rate in stem cells in genes involved in the regulation of epigenome as have been reported in functional normal HSCs that evolves to preleukemic and acute myeloid leukemia cells (Corces-Zimmerman et al. 2014). Variation in epigenomic patterns may trigger genomic instability and clonal selection in aged tissues. Epigenetic changes may also be a consequence of mutations, and this fact could be considered as a variant of a mutated phenotype, because the disturbances over epigenome control genes may propagate not as an increase in DNA sequence changes but of altered epigenetic imprinting that at last may lead to genomic instability in a self-propelled process.

Another pathway toward aneuploidy can be related to changes in methylation. Human malignancies are related to changes in methylation patterns that include hypermethylation of CpG islands, often related to gene promoters that could silence genes that affect cell cycle, DNA repair, apoptosis, and genes that are frequently

mutated in cancer cells. These hypermethylated genes that results permanently in on "off" state notably codes for developmental transcription factors that prolonged the stemness and increases the probability of malignant transformation. Hypomethylation linked to cancer may also be present and is known to increase genetic instability but also naïve pluripotency in ESCs (Leitch et al. 2013; Pfeifer 2018).

It must be stressed the role chromosome passenger proteins, as Aurora centrosomal kinases or the already mentioned, cycle-associated molecule, survivin, proteins that when dysregulated can induce aneuploidy or tetraploidy (Nguyen and Ravid 2006). In the inner centromere, a membraneless organelle is located, the so-called chromosome passenger complex, that is needed for the organization and regulation of mitotic movement of chromosomes (Trivedi and Stukenberg 2020). There are probable driver genes that may increase the occurrence of chromosomal aberrations and the concern on neoplastic potential of therapeutic candidate stem cells (Ben-David 2015).

Cell Origin as Source of Genome Instability

Cell origin for stem cells therapy, including those that are isolated, expanded, and utilized directly or after induction of pluripotency, must be considered as a differential source of instability. There are differences in genetic stability that are inherent to stem cell type, isolation, handling, and culture procedures while other arises from reprogramming factors utilized in the IPSCs obtainment.

Peripheral blood mononuclear cells (PBMCs) and hematopoietic stem cells HSCs can be reprogrammed to IPC. There is a large list of sources where cells to be reprogrammed can be obtained like urine, blood, cord blood, amniotic fluid, menstrual blood, teeth pulp, keratinocytes, fibroblasts, among others. These are differential in their tendency toward genetic instability, an example are human dental stem cells that are very unstable, showing around 70% of structural and numerical aberrations like polyploidy, aneuploidy, aberrations that increases during culture (Duailibi et al. 2012; Glicksman 2018).

In the next section, evidences for genetic instability in three types of cells whose relevance is out of discussion will be summarized.

Adipose Stem Cells (ASC)

Adult stem cells are the most relevant source of cells so as to be used in cell and tissue regenerative therapy, and those stem cells can be obtained with minimum-risk procedures (Miana and González 2018). ASCs are a source of MSCs with therapeutic properties, isolated from the stromal vascular fraction in adipose tissue. One of the main features of ASCs is their greater genetic stability when compared with other stem cell types.

Adipose stem cells have been repeatedly considered as those cells that better conserve its genetic stability. ASCs isolated from the stromal vascular fraction are

free of chromosomal aberration through G banding analysis, after during five passages in culture (Debnath and Chelluri 2019). There are evidences of negative selection of aneuploid clones of ASC that have been in culture for 6 months. A single subclone showed alterations in telomeric and subtelomeric regions at early passage, but were absent after prolonged culture time (Meza-Zepeda et al. 2008). Other studies reinforced the notion that ASC are stable after long culture time. Evaluation of chromosome stability with FISH probes for chromosomes X and 17 yielded 97.8% of diploid cells after 35 population doublings (Grimes et al. 2009). There are also evidences that points to a stable proportion of aneuploid subclones that could be increased in ASC cultures up to 16 passages (7.1%) and further during senescence (19.%), but without acquire tumorigenic capacity as was tested in nude mice (Roemeling-van Rhijn et al. 2013). Emergence of polyploidy may enhance cancer cell proliferation as have been reported in a murine model, where the polyploid ASCs was more efficient promoting breast tumor growth and metastasis than ASCs, derived from visceral adipocytes with a normal karyotype (Fajka-Boja et al. 2020). It must be stressed that quiescence of MSCs favors the generation and accumulation of genetic alterations that may result in genetic instability, especially after genotoxic insults. Genetic instability may induce senescence, apoptosis, and functional impairment that diminish or abolish the therapeutic efficacy of MSCs (Banimohamad-Shotorbani et al. 2020). It is a reason for the development and standardization of procedures intended to limit the exposition to DNA damaging procedures during cell isolation and expansion.

Genetic stability is a central aspect that must be considered when discussing therapeutic application of those cells and is one of the reasons besides their accessibility and abundance to consider these cells among the best suitable for medical uses (Miana and Prieto 2018).

Embryonic Stem Cells

Human pluripotent stem cells (hPSCs) include hESCs and human induced pluripotent stem cells (hiPSCs). hESCs are prone to acquire focal genomic abnormalities in culture, changes that seems to be nonrandom in origin (Lefort et al. 2009; Yoshihara et al. 2017). When considering the genetic stability in ESC, there is a consensus on the higher prevalence in genetic abnormalities like sub-chromosomal copy number variations (Laurent et al. 2011). However, in murine models, the mutation frequency in mouse embryonic stem cells (mESCs) have been estimated in one hundred percent lower than their mature counterparts from the same source, and this could be interpreted as the consequence of a better DDR mechanism so as to compensate their intrinsic genetic instability (Lo Furno et al. 2016). Blastocyst seems to sacrifice the proofreading functions during DNA synthesis in order to attain a faster cell division. In cultured ESC, there is a shortened G phase and interphase, consequently errors during replication occur more frequently (Henry et al. 2019).

The p53 pathway is affected in ESCs, where in spite of the overexpression of p21 RNAm, the P21 protein itself is not produced, hence, this partially abolished

response permits replication errors to continue (Dolezalova et al. 2012). After DNA damage in ESCs, p53 binds to the pluripotency factor NANOG's whose inactivation promotes the progress of ESCs to differentiation. Differentiation induction is part of a mechanism intended for the enhancement of genetic stability in ESCs (Lin et al. 2005). It has been reported that p53 knockdown provokes the downregulation of NANOG and Oct4 favoring differentiation. But in the absence of damaged DNA, p53 contributes to maintenance of NANOG expression and hence favors self-renewal in ESCs. This is an example of the complexity of the relationship between DNA damage, DDR, and stemness in these cells (Abdelalim and Tooyama 2014).

Another characteristic is the increase in genetic abnormalities at different resolution levels as the number of passages in culture become higher (Maitra et al. 2005). There is a report on the early development of sub-chromosomal and chromosomal aberrations since the passage number 5, and this result points to a strong genetic instability dependence on the number of passages (Bai et al. 2015). In an excellent study that evaluated several culture conditions on genetic instability, the frequency of genetic aberration increased significantly after 80 passages that were performed mechanically in hESCs and hIPSCs (Garitaonandia et al. 2015). In newly obtained ESC, there are earlier reports on the acquisition of deletions after reprogramming; those abnormalities duplicated its frequency as the number of passages increased (Laurent et al. 2011; Ben-Yosef et al. 2013; Garitaonandia et al. 2015).

Human unrestricted somatic stem cells (USSCs) represents a group of adult somatic stem cells CD45-negative population derived from human cord blood, and these cells grow adherently and maintain pluripotency to develop into cells from three layers along the expansion period without spontaneous differentiation. USSCs cultured on chondrogenic medium showed a conserved normal karyotype after expansion to 10^{15} cells. Those cells have shown to sustain their genetic stability until passage 6, expressed in normal karyotypes and relatively conserved telomere length (Kögler et al. 2004), that is extremely important for pluripotency, self-renewal, and genomic stability (Liu 2017). USSCs can be grown to make feeder layers for ESC cultures (Keshel et al. 2012).

ESCs cultured on conventional feeder layer compared to those grown in feeder-free cultures show more genetic stability. Feeder-free culture more often results in aneuploidy, chromosomal or sub-chromosomal mutations. In a pioneer study on ESCs karyotype stability, the modality of feeder-free culture encompassed rapid changes that involved gains in chromosomes 12 and 20. Those aberration appears after passages 17 and 21, as was demonstrated in two lines (HS181and SHEF-3) and a gain in chromosome 14 after passage 10 in the SHEF-3 line. The proportion of cells with trisomy 12 increased during culture time that was interpreted as a selective advantage. The same cells lines grown in feeder cultures remained genetically stable after 185 passages, irrespective if the passage method was mechanical or enzymatic. Surprisingly, SHEF-1 that conserved genetic stability after 185 passages in feeder layer culture did not suffered any chromosomal changes when transferred to a feeder-free culture for 30 additional passages. Different susceptibility between different cell lines must be cautionary considered for ESC as therapeutic candidates (Catalina et al. 2008; Guo et al. 2018).

In the cell line HS181, other authors have shown that chromosomal aberrations appeared in all the cells that became adapted to growth directly on plastic surface. Chromosome X trisomy, deletions in 7q11.2 and I (12) p10 were the nonrandom aberration detected. However, the greater survival and growth in feeder-free conditions was accompanied by reduction in pluripotency (Imreh et al. 2006). Anyway, the ability exhibited of certain cells bearing trisomies to properly differentiate is a real warning issue, because this aneuploidy persistence poses a risk for tumor development if those cells able to thrive and differentiate, are inoculated as a therapy procedure (Zhang et al. 2016; Bach et al. 2019).

Similar results with feeder layers points to a protective effect of culture, upon feeder layers or extracellular matrix (ECM) proteins, on karyotype stability (Escobedo-Lucea and Stojkovic 2010; Lee et al. 2012) not only for ESC but also in IPSCs (Nakagawa et al. 2014; Ghasemi-Dehkordi et al. 2015), mESCs cultured on feeder layers made of hUSSCs maintain normal karyotype (Keshel et al. 2012). Feeder layer made up of bone marrow MSCs maintained the adequate phenotype in ESCs and absence of aneuploidy (Lee et al. 2012). The use of smaller feeder layer like the *microdrop culture method* have been applied for the generation of bovine ESCs with favorable results regarding pluripotency and the conservation of a normal karyotype (Kim et al. 2012a, b). In murine models, mouse stem cells maintain its genomic integrity during long-term cultures on a feeder of mouse embryonic fibroblasts derived matrices (MEFDMs) but non when cultured on gelatin-coated substrates (Kim et al. 2012a, b; Sthanam et al. 2017). There is a recent report of bovine stem cells that with the introduction of a defined culture substrate (N2B27) have been able to maintain a normal genetic constitution over 35 passages without the use of mouse embryonic fibroblasts feeder layers. It is a demonstration that the use of feeder layers with its potential inconveniences can be circumvented with more refined approaches in the culture conditions that include defined medium like N2B27 (Soto et al. 2021). Feeder layers favors genetic stability but this goal can be reached even without that. The passage methods as have been already mentioned is another source of karyotype changes In ESC, the impact of mechanical procedure compared to that of enzymatic treatment remain controversial. Some authors have reported that irrespective of the techniques employed, genetic instability may be detected (International Stem Cell Initiative 2011; Tosca et al. 2015). Cell passage in culture based enzymatic detachment is related to greater genetic instability when compared with the mechanical one (Garitaonandia et al. 2015). However, the use of a mixture of proteolytic enzymes, including both trypsin and collagenase activity (Accutase), does not require inactivation, hence reducing handling, and did not induce abnormal karyotype in ESCs cultured under feeder-free conditions (Kim et al. 2012a, b).

In this respect, there are contradictory reports on the beneficial effects of Accutase use in cell passages. As an example, when ESCs and IPSCs were evaluated over 100 passages, those cells cultured showed deletions involving p53 locus and less activation of p53 downstream genes. But in spite of the use of Accutase-based passage, deletions were larger and appeared earlier than that in mechanical passages. Duplications and deletion involving chromosomes 20, 12, and 17 were consistently detected with a higher frequency in ESCs and IPSCs that undergo enzymatic

passage, and karyotype mosaicism was also detected (Holm et al. 2013; Garitaonandia et al. 2015; Amir et al. 2017). Even when compared with the less harmful Accutase, the mechanical cell detachment is the best choice against the induction of genetic instability.

Even with this supposedly less harmful passage procedure, a relationship between passage number and p53 mutations is noteworthy (Holm et al. 2013; Amir et al. 2017). Involvement of p53 mutations in ESCs genetic disturbances in culture was also demonstrated when 5 cell lines resulted positive for p53 dominant negative mutations, afterward when 140 lines were analyzed, from that mutated, the allelic fraction in 3 cell lines increased its prevalence to more than 50%, signifying what was interpreted as selective advantage for p53 mutations bearing cells. The authors stressed the fact that hPCS have been used in spite of containing cancer mutations in p53 (Merkle et al. 2017). Mutations in p53 were detected in cells cultured with several media, substrates, and using a variety of passage procedures. That is interpreted as evidence in the sense that current culture conditions exert a positive pressure for p53 mutations. This gene should be in the focus for the analysis of genetic adequacy of stem cell lines for therapy especially when culture goes through any intervention involving the existence of bottle neck population period, when the mutation may affect a greater proportion of cell populations (Merkle et al. 2017).

In hESCs, the function of p53 is uncertain; some evidences support the notion that p53 is responsive to DNA damage as occurs in adult cells, while others have shown the absence of downstream activation of genes like p21 that build up the DNA damage response. An alternative effect of genotoxic insult is characterized by apoptotic induction that relies on p53 upregulation and relocation in mitochondria, without involvement of typical genes from p53 networks that are activated if the damaged cells are induced toward differentiation (Tichy 2011).

In mESCs, cell cycle is shorter with a predominance of cells in S phase, and those cells lack G1/S checkpoint. Chk2 that phosphorylates and stabilizes p53 also acts over the cycle regulator phosphatase Cdc25a that cannot remove phosphate from Cdk2. This phosphorylated state make cell unable to proceed into S phase. However, in mESCs, miss localization of p53 does not favor Cdc25 abrogation and damaged cell may enter S phase and continue the cycle. In mESCs, there is no G1 checkpoint. However, in human ESCs, the ability of p53 to activate a DDR response and G1/S checkpoint depends on the nature of the genotoxic stimuli, and it has been shown to respond when cells are exposed to UV but not against ionizing radiation (Tichy 2011).

Human ESCs, human nuclear transfer ESCs parthenogenetic hESCs, and iPSCs comprise the hPSCs that are suitable for therapeutic applications and also show susceptibility to genetic and epigenetic instability that makes mandatory for in-depth evaluation before their use (Simonson et al. 2015). Aneuploidies in hPSCs involves chromosomes whose numerical aberrations are not only incompatible with life but also resembles that chromosomal gains and losses found in human embryonic carcinoma cells, in what can be interpreted as a hint for their tumorigenic potential (Peterson and Loring 2014). However, there are results that points to the fact that the use of cells from secured sources is an example of how the genetic instability can be

restricted in dependence of good manufacturing practice (GMP) for the obtainment of standardized ESCs. There are experiences with ESCs that after in-depth evaluation of its genetic stability did not express variations at chromosomal or sub-chromosomal levels neither gained mutations in culture (Canham et al. 2015).

Duplications and deletion involving chromosomes 20, 12, and 17 were consistently detected. Additionally, chromosome 12p duplication is known to bear the loci for the pseudogene NANOGP1 and NANOG gene, a known pluripotency marker is also a hallmark for teratocarcinoma. Chromosome 20 duplication is associated with higher expression of Bcl-xL that may drive survival advantages (Nguyen et al. 2014; Clark et al. 2004).

Base excision repair is active in ESC and IPC with high activity irrespective of the parental source from which they were derived. Regarding DSB repair, both stem cells show more active high-fidelity HR, which is a requisite for the conservation of pluripotency and the ability to develop a complete organism, while upon differentiation, they begin to rely on error-prone NHEJ (Tichy 2011; Tilgner et al. 2013; Zhang et al. 2018a, b). Lest say that the accuracy of DNA repair is decreasing along with the greater differentiation stage.

Induced Pluripotent Stem Cells

IPSCs were obtained, as we mentioned before, through the introduction of genes or reprogramming factors with the purpose of reversing their development program to resemble ESCs. IPSCs are known for their propensity to aneuploidy, flaw that is more evident after a long culture period. When genetic instability of IPSCs is analyzed it is often difficult to stablish a a clear separation from the instability that is inherent to ESCs. Those cells, in spite of their differences, exhibit a high degree of aneuploidy. IPSCs are originated in blastocysts where aneuploidy is frequent. There is evidence for auto-correction of aneuploidy in ESCs, but the process is still poorly understood (Bazrgar et al. 2013).

Developed ESCs exhibits mainly chromosomal gains in contrast to that of blastocysts which experienced both losses and gains, those ESCs chromosomal gains seems to be related to selective advantages. There are three main causes for genetic alterations in IPSCs: progenitor cells with preexisting mutations, DNA changes that arise during reprogramming, and those mutations that appears de novo during proliferation in culture (Tichy 2011). There are another causes of aneuploidy like supernumerary centrosomes that are often found in hESCs derived from human zygotes and cleaved embryos that aroused from disturbed fertilization, characteristically, the proportion of hESCs bearing the anomalies is lower than that in their earlier progenitor (Gu et al. 2016).

IPSCs are very similar to ESCs but are not identical; those are produced departing from cells treated in a way that their gene expression is altered, as well as its development route that changes the original cell into another cell type. Procedures to develop IPCs are nuclear transfer, cell fusion, or transcription-factor transduction. In the last approach, IPSCs from reprogramming is a consequence of unbalance

stoichiometry of the transcriptional regulators present in the cell through transfection of reprogramming genes that surpassed a threshold needed for the conversion to a pluripotent cell (Amps et al. 2011; Tichy 2011; Henry et al. 2019).

There is growing evidence that genetic stability and pluripotency are regulated by transcriptional changes in differentiated and undifferentiated states, and is known that iPSCs are extremely prone to genetic instability, a problem that is favored by inefficient reprogramming (Paniza et al. 2020). In other words, the fidelity of genomic reprogramming is affected due to the persistence of "epigenetic memory" that reflex the fact that the epigenetics marks, inherited from the progenitor cells (Tichy 2011; Basu and Tiwari 2021). These epigenetic pattern that extended beyond reprogramming into the life of iPSCs may affect genes responsible for the maintenance of DNA integrity and euploidy. Those epigenetics modifiers are microRNA, regulators of DNA methylation, histone chemical marks, and ATP-dependent chromatin remodelers (Simonsson and Gurdon 2004; Hassani et al. 2019). There are positive and negative feedbacks, between DNA sequences expression and those modifiers, in a way that have been compared with that of the Operon-lac, described in prokaryotes. Maintenance of pluripotency also depends on those feedback loops between epigenetic landscape of DNA sequences and pluripotency genes, like the Yamanaka factors. Persistence of epigenetic memory is considered as an important contributor to genetic and epigenetic disturbances in iPSCs (Yamanaka and Blau 2010; Tichy 2011; Di Giammartino and Apostolou 2016; Pelham-Webb et al. 2020).

However, other authors have not found a relationship between methylation patterns and genetic instability in IPSCs. Their results pointed to inefficient reprogramming as a cause, leading to incorrect DNA replication and replicative stress, and the evidence reveals impaired DNA replication with less origins and a high frequency of strand breaks in iPSCs (Jekaterina et al. 2020; Paniza et al. 2020).

IPSCs reprogrammed carries a mutational burden accumulated during the previous somatic cell life besides the fact the event of reprogramming causes a severe disturbances in DNA chromatin homeostasis. The proportion of cells that experienced a correct reprogramming is generally low and interestingly may be increased if p53 is inactive, and this poses a greater risk for iatrogenic induction of a tumor, if cells reprogrammed for therapy eluded the response against DNA damage and mutation (Tichy et al. 2011; Amir et al. 2017). There are evidences suggesting that iPSCs genetic stability is poor, because of a lack of generation of an adequate DDR, in spite of the elevation of expression of DNA repair genes. A dissociation between expression and production of DNA repair proteins have been invoked as was already mentioned in reference to p21. There is strong evidence that genomic stability is much lower in iPSCs than in ESCs due to less DNA repair fidelity (Zhang et al. 2018a, b). The mutation frequency exhibited by ESCs is minor than that in adult somatic cells and even lower than IPSCs, it could be a reflection of the more efficient mechanism that evolved in embryonic cells, whose correct functioning is essential for the development of the embryonal tissues and organs. The fact that mutations could be transmitted to the next generation places the mechanism for DNA damage detection, repair, or amelioration in ESCs as a safeguard of the genetic integrity of the species (Adiga et al. 2010). However, both cell types exhibited a combination of

additional mechanisms against genotoxic insults, a hyperactive and rapid DNA repair, elimination of cells with damaged DNA and differentiation in order to withdraw the mutated cell from the stem cell compartment. Another defense mechanism in ESC and IPSCs is based on lowering the number of mitochondria in order to limit the reactive oxygen generation (one of the main causes of endogenous DNA damage), while genes for antioxidants become overexpressed (Forsyth et al. 2008; Maraldi et al. 2021).

Effect of Reprogramming Methods on Genetic Instability

Reprogramming based upon virally integration of the factors is also an event of insertional mutagenesis that elicit several abnormalities in the recipient genome; moreover, some of the reprogramming factor expressed in the IPSCs provokes genetic disturbances like CNVs, changes in the regulation of imprinted genes, point mutations, wrong methylation patterns, as well as chromosomal aberrations (Kang et al. 2015).

Those are enough reasons to sustain and justify the search for non-integration based reprogramming procedures. Among those methods that do not rely on the integration of foreign sequences are: episomal vectors, plasmids transfection, vectors derived from Sendai virus, adenoviral vectors, synthetic mRNA, miRNA, plasmid transfection, minicircle vectors, transposon vectors, liposomal magnetofection, protein transduction, and small molecules. The non-integrating reprogramming procedures are less efficient and limited to certain types of cells like fibroblasts, but their impact on genetic instability in newly developed IPSCs is substantially minor (Goh et al. 2013; Steichen et al. 2014; Beltran et al. 2020; Schlaeger 2018; Haridhasapavalan et al. 2019). IPSCs are the basis of a strategy to obtain an unlimited source of renewable cells to be utilized in autologous transplants. These cells, however, show a high degree of genetic instability that is related to several causes; among them, the reprogramming procedure is one of undeniable importance.

hiPSCs genetic stability is mostly influenced by the reprogramming method, which may be integrating vector-dependent and vector-independent methods. It is accepted that integrated methods causes more genetic instability as expressed in CNV, SNP, and mosaicism. A study of genetic aberrations in ESCs, progenitor cell lines, and IPSCs line versus IPSCs non-integrating lines through Affymetrix Cytoscan HD array resulted in CNV 20 times larger in average than those found in non-integrating IPSCs lines. CNV, SNP, and mosaicism were more abundant in integrating IPSCs than in progenitor cells, ESCs, and non-integrating iPSCs lines (Kang et al. 2015). However, there is another view point. Some researchers have found at least two CNV in progenitor cells, similar to those present in the reprogrammed iPSCs, and it is interpreted as the preexistence of low level CNV mosaicism in progenitor cells that are more often detected in iPSCs, due to their clonal expansion that lead to CNV enrichment and not necessarily new CNV linked to reprogramming (Abyzov et al. 2012).

Mutations Induced During Reprogramming

In a study that involved 22 hIPSCs reprogrammed with different procedures, 5 mutations were detected, some of them involving genes that participates in cancer development. The wide exome sequencing yielded six mutations per exome. Four new random mutations appeared as culture time was longer. Some mutations were originated during reprogramming while others, approximately the half, were preexistent in progenitor cells although at low frequencies (Gore et al. 2011). On the contrary, some potentially lethal mutations were only present during limited periods that are related to differentiation process (Fischer et al. 2012). It have been demonstrated that in early cultures of both hESCs and hiPSCs, deletions and loss of heterozygosity (LOH) are generated with a high frequency while duplications are more susceptible to occur during long-term culture. The type of genetic changes seems to be related to derivation process (Ben-Yosef et al. 2014). There are several studies that have proven that reprogramming is in itself a procedure that causes mutations and chromosomal aberrations in the iPSCs (Sugiura et al. 2014; Liu et al. 2020).

Different strategies were adopted in order to dissect the precise origin of those mutations. Most of mutations arose early during reprogramming and were not clonal from a parental origin. The effect of reprogramming procedures, especially those that are integrative, is recognized as risky and poses a severe hurdle on the therapeutic used of iPSCs. Those facts have fueled the research to establish protocols that minimize the reprogramming related mutations in IPSCs (Yamanaka and Blau 2010; Haridhasapavalan et al. 2019; Yoshihara et al. 2019; Schaefer et al. 2020).

In spite of the fact that there are mutations that exist prior to reprogramming in the starting progenitor cells, there are others that appear during reprogramming process and afterwards in culture. These mutations can be selected positively or negatively. The allegedly function of those mutations is that they could be involved in reprogramming process itself but unfortunately those changes also could be related to cancers, with which mutation pattern are similar to reprogramming related mutations (Gore et al. 2011). The fact that insertional based reprogramming procedures often induce de novo mutations, that not necessarily are negatively selected in culture, has stimulated the search for alternative reprogramming methods, such as those based on expression plasmids, Sendai virus vectors, and episomal plasmid vectors. An array of DNA-free reprogramming methods, including protein-based, mRNA-based methods, have also been developed (Yamanaka and Blau 2010; Basu and Tiwari 2021).

The relationship between reprogramming and generation of mutations is also thought not as a consequence of the introduction of reprogramming factors, but to the process of integration in the genome of receptor cell. There is a growing number of reports on that line. The nuclear transfer procedure in mouse embryonic and tail-tip fibroblasts yielded fewer mutations than those that resulted after retroviral transduction. Nuclear transfer generated 80% less mutations than retroviral transduction (Araki et al. 2017). Integrative methods induces more mutations but there are differences in their mutagenicity, like that based in Sendai versus Lentivirus, the

alternatives within integrative methods is also opened (Sobol et al. 2015). Moreover, there are voices in dissidence that considers that reprogramming method does not affect gene expression in iPSCs (Trevisan et al. 2017).

Besides the circumstance that reprogramming methods exerts an influence over the genetic and epigenetic stability in iPSCs, there is also another particular issue that must be addressed: the reprogramming factors or genes itself. Reprogramming factors, currently in use like OCT4, SOX2, KLF4, c-MYC, NANOG, and LIN28 are involved in several malignancies as drivers of tumorigenesis and biomarkers of cancer severity and evolution. Reprogramming factors are at the same time oncogenes, inducers of replicative stress and genomic instability in resultant iPSCs, and this is particularly relevant when c-Myc is included as a reprogramming factor (Pasi et al. 2011; Jeter et al. 2015; Kuo et al. 2016; Lamm et al. 2016; van Schaijik et al. 2018).

Shimada et al. (2019) exposed to ionizing radiation IPSCs to characterize transcriptional changes leading to pluripotency and genome stability maintenance under genotoxic pressure. The endpoints were DNA DSB repair, cell cycle checkpoints, and apoptosis in fibroblasts, iPSCs, and neural progenitor cells (NPCs) derived from iPSCs. DSB were repaired more efficiently in fibroblasts, followed by neural progenitor, while in IPCs remained a higher percentage of DSB, as was demonstrated through detection γ-H2AX foci. In IPSCs, apoptosis-related genes like p53, CASP, and BID were overexpressed as well as apoptosis markers increased. Those results points to a relevant role of apoptosis in IPCs, erasing DNA damaged cells, contributing in that way to genomic maintenance of undifferentiated cell populations exposed to genotoxic insults.

Not only those genes that constitutes reprogramming factors have exerted different degree of influence in the resulting IPCs. Genes involved in remodeling of nucleosomes through deacetylation like Mbd3 are determinant in reprogramming efficiency (Jaffer et al. 2018). Another is CHK1 whose overexpression increases the efficiency of the procedure and the oocyte factor Zscan 4 that contributes to maintaining genomic stability, limiting sister chromatid exchanges and promoting telomere elongation in mESCs. Genes like developmental pluripotency-associated protein-3 (DPPA3) and (NPM2) nucleoplasmin-2 that are involved in Zscan4 regulation and stemness maintenance cooperate when co-transfected with Yamanaka factors in the genetic stabilization role in murine embryo fibroblasts (Jiang et al. 2013).

Another relevant group of genes that are determinant in the success of reprogramming belongs to DNA repair mechanism, like XPA from NER, Brca1, Brca2, and Rad51 (González et al. 2013), that can be interpreted as an evidence of the need of an intact HR mechanism during reprogramming. Deficiencies in DSB NHEJ repair causes both a reduction in reprogramming efficiency and increases genomic instability. Functional impairment of ligase IV (LIG4) and Cernunnos/*XLF*) that participate in NHEJ also sustains the role of DDR in the reprogramming (Tilgner et al. 2013; Turinetto et al. 2017).

Sirtuins (SIRT), a group of type III histone deacetylases that change chromatin conformation in specific sites, have been invoked as participants in DSB repair.

There is a link between SIRT function and poly(ADP-ribosylation) in cells with damaged DNA. There are reports on the effect of SIRT inhibition that results in a very profound reduction in DNA-PK-independent nonhomologous end joining, a backup pathway (B-NHEJ). NHEJ is active in stem cells and the fact that SIRT inhibitions reduces this activity is in concordance with those reports about SIRT expression as relevant for the genetic stability in stem cells (Wojewódzka et al. 2007; Chen et al. 2017; Fang et al. 2019; Jeske et al. 2021). In line with the previous assertion, SIRT1 participates in the regulation of mitochondria activity in ESC (Ou et al. 2014), and consequently in their ROS production and ability to damage DNA.

NAD+ levels are reduced in aging mice that in turns reduces SIRT1 function and decreased NAD+. The lowered levels of NAD+ inhibits PARP1 because a protein DBC1 that must combine with NAD+, but if NAD+ it is reduced, DBC1 is favored to combine with PARP1, that diminish its activity resulting in more DNA damage. In this circumstances, both NHEJ and HR are also reduced. The so-called NAD+/PARP1/SIRT1 axis explains a connection between NAD+ levels and DNA damage. These links, that are relevant to explain age-related changes in the ability to respond to genotoxic insults, bring another two molecules that are involved in genetic stability in stem cells, NAD+ and SIRT family (Mendelsohn and Larrick 2017). In human MSCs, mitochondrial NAD+ restoration delays aging and replicative senescence in culture (Son et al. 2016).

Moreover, SIRT have been demonstrated as a factor that alters IPSCs reprogramming through metabolic changes in the cells. This is a gene family that may be amenable for modulation in order to improve reprogramming (Shin et al. 2018). Another family of enzymes that is crucially involved in stem cell renewal, proliferation, and differentiation is that of NADPH oxidases, which mediate several basic cell processes trough modulation of redox status in embryo cell in their natural environment and also in culture expansion for therapy purposes. Oxidative stress and antioxidant capacities are antagonists in the delicate oxidative homeostasis; NOX enzymes may modulate protein activities through ROS signaling that changes stem cell main characteristics that are relevant for their clinical applications, i.e., stemness, homing, and genetic stability (Maraldi et al. 2021). NADPH oxidases, like type NOX4, are present in mitochondrial membrane, the endoplasmic reticulum, and nuclear membrane (nNOX), the last modify the expression of stemness factors Oct4, SSEA-4, and Sox2, and modulates DNA damage (Maraldi et al. 2021).

Culture Conditions, the Passage Number

Stem cells are not abundant enough to be utilized straightforward in therapy, so they need to be expanded in culture. Culture comprises an array of steps and conditions that make each one a probable source of heterogeneity and further variation. Donor's individual characteristics, cell origin, culture medium and supplements, oxygen pressure and different protocols for cell detachment, passages, and environmental radiation levels, are factors that could be summarized as a need for greater

standardization of the whole process from cells isolation to transplantation. This shortage should be expected in a growing science field, where the development of consensus protocols is urgently needed in order to limit variability and contribute to achieve more homogeneous therapeutic cells.

Culture conditions have been invoked consistently as a main contributor to genetic instability. Different types of stem cells are prone to exhibits and increase in chromosomal and sub-chromosomal abnormalities (CNV, indels), aneuploidies, and de novo mutations. Culture as a necessary procedure for cell expansion involves critical steps that may affect genetic stability, among them there are the culture medium composition, including the option of defined mediums, the normoxic or hypoxic conditions, the freeze-thaw and the passage techniques, the addition of substances that may affect DNA repair capacity, the presence or not of feeder layers, and the passage number among the more relevant (Mitalipova et al. 2005; Narva et al. 2010; Di Stefano et al. 2018; Henry 2019). Passage procedure involving single-cell or small-clump passaging is considered as relevant source of genetic changes (Bai et al. 2015). Karyotype abnormalities and copy number variations does not only occur during long-term cultures. Those genomic changes may appear early within a number as low as five passages in hESCs. Those alterations are detected after enzymatic treatment for cells detachment, as was already mentioned; enzymatic digestion induces genomic instability in hPSCs, even at the first passages. Sub-chromosomal abnormalities arise earlier than those revealed in the karyotype and were associated with higher frequency of DSB (Bai et al. 2015; Assou et al. 2020).

Amniotic fluid-derived MSCs are less prone to genetic damage than BM-hMSCs, and one of the reason is the better repair response to DNA damage in the former after the same genotoxic challenge (Alessio et al. 2018). A report on genetic instability in human ASC under normoxic (21% oxygen) and hypoxic conditions (1% oxygen) points to a greater susceptibility of ASC versus BM-hMSCs to impaired DNA repair as evaluated by γH2AX signaling, the authors recommended hypoxic as the normalized culture conditions for BM-hMSCs (Bigot et al. 2015). Oxygen conditions in culture should be maintained hypoxic, but the best choice must be to match the gas pressure to the cell type. Stability in the oxygen flow must be secured in order to limit those fluctuations that is accompanied by ROS generation and DNA damage (Goto et al. 2014; Oliveira et al. 2014; Testa et al. 2016).

There are proposals for enhancement of culture conditions through development of standardized platforms based in more efficient bioreactors at a greater production scale that should result in lower number of passages. Development of defined culture media that overcome the troubles associated to chromosomes abnormalities that are more often found when serum free or poorly defined media is utilized. The presence of genotoxicants during culture is been addressed (Talib and Shepard 2020). Along the chapter we have mentioned those aspects relating culture conditions and genetic instability, and hence in this section, we will focus on the proposed solutions that addressed the enhancement of genetic instability trough standardization of culture, media components, manipulation, and even selection of progenitor original cells in order to favor the homogeneity as well as stability.

There have been proposed that cells to be used for therapy must be restrained to the first passage number, according to specific cell type, and culture media. The proposed passage number that should guarantee greater safety regarding genetic stability ranged from two to six for hESC (Merkle et al. 2017), to four passages in BM-hMSCs (Binato et al. 2013), and also four passages in MS cultured in DMEM (Yang et al. 2018). Another group found the limit for BM-hMSCs in passage three (Zhang et al. 2007).

Greater stability in long-term culture have been reported for BM and adipose-derived stem cells that remained genetically stable up to passage 20 in α-MEM/20% FBS (Izadpanah et al. 2008). There is another report on the suitability for BM-hMSCs that until ten passages conserved karyotype stability as well as their secretory ability, needed for their paracrine effects (Choi et al. 2010). There is a lack of consistence between different groups in finding a passage threshold of security, for instance in another work with the same cell type resulted only in two passages secure interval (Borgonovo et al. 2014). Similar results have been reported by von Bahr et al. (2012), regarding also the therapeutic efficiency declining after two passages for BM-hMSCs.

ASCs yield from adipose tissue are about 500-fold times greater than from bone marrow, and this allows less expansion culture time for adipose-derived stem cells, which is an important advantage (Jeske et al. 2021). Additionally, ASC can be maintained in culture during greater time periods with less risks of decay in therapeutic properties (Danisovic et al. 2017; Jeske et al. 2021). ASC cultured during periods of 6 months maintained their genome stability evaluated through in deep high-resolution techniques, and interestingly the authors remarks that in a negligible subpopulation, certain aberrations arose in early culture, but were further eliminated, even reducing the already low probable risk (Meza-Zepeda et al. 2008).

Moreover, in the search of the secure number of passages for therapy use, there are reports of adipose tissue-derived mesenchymal stem cells that shows chromosomal stability, even after scrutinized through high-resolution karyotyping, until passage five, when cultured in low-glucose Dulbecco's modified eagle medium (L-DMEM) (Debnath et al. 2019). ASC respond with less senescence during culture, a feature that is considered as a result of higher sensitivity in NAD+ –SIRT axis (Jeske et al. 2021). Adipose-derived stem cells remain genetically stable over long periods in culture as well as keeping their pluripotency, differentiation capacities, and immunomodulatory properties; characteristics that make ASC a central protagonist in cell-based therapy (Grimes et al. 2009; Blázquez-Prunera et al. 2017; Patrikoski et al. 2019).

Human umbilical cord derived MSC (hUC-MSCs) experienced changes in expression involving genes related to chromosome stability and segregation altogether with senescence morphology after passage number 15 in DMEN 10% FCS, those changes were paralleled with decreasing differentiation and regeneration abilities (Zhuang et al. 2015). Other authors report that (hUC-MSCs) are massively selected since the beginning of culture expansion, a process that results in a more homogeneous population (Selich et al. 2016), exhibiting a greater fitness. However, there is a consistency in the results indicating that mesenchymal stem cells isolated

from adipose tissue results in more genetically stable cells than their BM counterparts. In a murine model, among the molecular features that could explain the differences between adipose and bone marrow derived cells, is significant the reduction in H-19 noncoding RNA in BM-derived cells compared with ASCs. This difference is considered as determinant to the proneness to polyploidy exhibited by BM-derived mesenchymal stem cells. In ASCs, there is also an involvement of p53 protein that is less active in BM-derived stem cells in keeping ASC diploidy (Ravid et al. 2014).

Given the intrinsic nature of variations in progenitor cells and during culture, the choice should be aimed toward a cultured cell, whose amount and nature of genetic changes does not compromise their therapeutic safety, and not to the total absence of genetic changes. This is a risky statement due to the stochastic nature of the previous changes that may lead to oncogene activation or LOH as well as to mutations compromising the differentiation and proliferation cell fate.

A parallel, more active, approach includes the use of mitogen-activated protein kinase (MAPK/ERK) inhibitors in stem cell culture. Inactivation of MAPK pathway with the MEK1/2 inhibitor PD0325901 often results in genetic instability in mouse cells. However, titration of this inhibitor from 1 μM to 0.3–0.4 μM or replacing it with another one may restore the epigenetic and genomic stability of mouse ESC, as well as their differentiation capacities. MAPK inhibitors are another alternative to be considered in the search for genome stability in stem cell culture (Di Stefano et al. 2018).

The Bystander Effect

The paracrine effect of an aneuploid cell over the surrounding cells through exosome is known as bystander effect. One relevant example regarding culture procedure is the influence over basement membrane of feeder cells, exerted by mitomycin C. This phenomenon is considered as a contributor to the induction of chromosome aberration in untreated neighboring cells. The bystander effect is under scrutiny so as to establish the contribution to aneuploidy induction by contiguity of stem versus feeder cells in different culture conditions.

Exposition to exosomes have been correlated to telomere shortening in non-irradiated human epithelial cancer cells, when exposed to irradiated conditioned medium (ICM) from irradiated ones, a phenomenon that increased with the passage number. In the progeny of non-gamma irradiated HSCs, from CBA/Ca mice, exposed to ICM, chromosomal instability similar to that of irradiated cells have been induced due to bystander effect (Lorimore et al. 2005). Exosomes from irradiated cells are proven responsible for bystander telomere effects. Proteins and RNA contained in exosomes have been found responsible for telomere-induced instability in nearby cell (Jella et al. 2014; Al-Mayah et al. 2017). Microvesicles have been previously identified as vectors for bystander effect between irradiated and nonirradiated human keratinocytes. Both exosomes (30–100 nm) and microvesicles (>100 nm) are involved in the transport of such molecules that induces

genetic instability in non-treated cells (Jella et al. 2014). Intercellular communication junction are also proven as passage ways for instability factors between cells like products resultant from oxidation (Klammer et al. 2015; Henry et al. 2019). There is a consensus respect to bystander effect as a factor that is relevant to maintenance of genetic stability in cells that are cultured in the presence of other that have been exposed to genotoxicants (Henry et al. 2019).

Instability Factors Related to Donors

Obesity

In MSCs obtained from adipose deposits in obese mice under oxidative stress produces an increase in DNA damage that may cause telomere shortening and activates genotoxic checkpoints. Oxidative stress linked to adipose inflammation compromise the expression of stemness genes and provokes more senescence and apoptosis. In ASCs obtained from old obese subjects, there is more DNA damage, less renewal, and therapeutic potential due to secretome impairment (Alessio et al. 2020). Similar results were obtained when obese diabetic subjects from India and nonobese control served as donors of hADSCs so as to evaluate the on ASDC functions. Proliferation was reduced, expression of pluripotency genes was altered as well as diminishing osteogenesis (Rawal et al. 2020).

Resistin, an adipokine that is increased in obesity, known for favoring insulin resistance, have been recently linked with lowering of the stemness of in cultured hADSCs from a healthy subject. Exposition to resistin obstacles differentiation to adipogenesis and reduced expression of several genes including CCAAT/enhancer-binding proteins (C/EBP)α and adiponectin in adipocytes and affect SIRT levels in derived in osteocytes (Rawal et al. 2021). Under hypoxic conditions, there have been observed a decrease in expression of DNA repair genes and consequently microsatellite instability. A reduction in the number of mitochondria accompanied with less production of ATP, simultaneously, a decrease in ATP that is independent from of O_2 concentration or number of cell passages. Several point mutations, including some in a wide range of tumors, were observed. This is one in many reports on the negative effect of hypoxia in stem cell genomic instability (Zhang et al. 2018a, b; Kaplan and Glazer 2020).

Donor Age

Senescence is a cell response to aging that is characterized by exhaustion of stem cells and chronic inflammation. However, there are considered coordinated processes that guarantee protein homeostasis. This processes include: synthesis, folding, ubiquitination, and proteasome degradation, and a disruption in this network results in toxic damaged proteins.

Also a senescence hallmark is the alterations in nutrient signaling and sensitivity to mitogens that is crucial in cell growth regulation. Cell growth is controlled by two opposite pathways, one that is anabolic called target of rapamycin complex I (mTORC1) and the alternative is the autophagy pathway that is catabolic.

mTORC1 reacts to several types of stimuli that integrates growth as a response for a multiplicity of metabolic situations, and among those effectors are growth factors, adenosine triphosphate, oncogenes, amino acids, and ROS. Senescence includes the breakdown of the control of genome, protein homeostasis, and nutrient sensing related growth (López-Otín et al. 2013; Carroll and Korolchuk 2018). Age-associated genome instability in stem cells is linked to mutations that accumulate in a stochastic manner or tends to increase in exponential dynamics of mutations. Emergence of driven mutations may lead to senescence or cancer in the context of reduction in several DNA repair pathways (Burkhalter et al. 2015).

Aging is expressed as a complex and gradual loss of functions that result in tissue and organ deterioration as well as the organism as a whole. Stem cells that are responsible for tissue homeostasis guarantee renewal and proliferation, experienced aging, and consequently accumulate damages that leads to senescence affecting their quality and usefulness to be utilized in cellular therapy (Kollman et al. 2001).

In this regard, age of donor subjects is important in the success of cell expansion. A series of reports support the negative effect of aging in stem cell suitability for therapy. IPSCs reprogramming was inversely correlated to donors age (Trokovic et al. 2015; Meng et al. 2020), as well as the yield of adipose MSC (Yamauchi et al. 2017). The negative impact of cells aging, affects differentiation efficiency, and paracrine immunomodulatory properties have been reported (Jin et al. 2017; Park et al. 2021). In a mixed model of murine recipients and human cells transplantation, the analysis of peri-infarct cortex treated with hMSCs from aged donors significantly reduced the efficacy relative to neurogenesis, vessel development, and anti-inflammatory secretory profile (Yamaguchi et al. 2018). A recent review that evaluated information on the links between vascular senescence, endocrine pro-senescence factors NSC responsiveness to vascular and blood factors, lead to the conclusion that aged stem cells declined in key processes like neurogenesis. These facts poses a warning signal against the use of elders as donors of NSC for therapy (Rojas-Vázquez et al. 2021).

Studies on the relationship between donor age and IPSCs genetic stability have demonstrated that cells from elder donors contained a higher number of genetic alterations than the younger. In a work IPSCs were obtained from blood samples from 16 subjects (age 21–100 years). Clonal expansion via reprogramming allowed exomic analysis that revealed a linear relationship between mutations and donor's age, and those mutation includes several related to cancer (Lo Sardo et al. 2017). There is a consensus on the greater genetic alterations and less suitability for stem cells derived from aged subjects (Kenyon et al. 2012; McNeely et al. 2020; De et al. 2021). This brings and implicit limitation for those autologous treatments in old patients that are being the subject of intense scrutiny that is continuously fueled by controversial results.

Alternatively, some author reported that adipose stem cells conserved the secretome without changes neither showed senescence or reduction in cells yield irrespective of donors age (Dufrane 2017). In this direction, there are reports in reversing age signature and fulfillment of successful reprogramming IPSCs from elder even centenarians subjects. Those studies that reports cell reversion of aged

phenotype in different kind of cells, like MSCs, NSCs, HSCs, and reduction of genomic instability hallmark, and remarkably epigenetic rejuvenation obliges to keep the possibilities opened to the acceptance of elder cells donors as the research on the continues (Lapasset et al. 2011; Rohani et al. 2014; Singh and Newman 2018; Li et al. 2020; Yu et al. 2020).

Toxic Habits and Diseases in the Donors

Smoking and alcoholism are habits that may harm donor cells, preconditioning then in such a way that affects the abilities for its use in therapy. Basal stem cells from airways of smoker subjects acquires gene signature of lung adenocarcinoma (Shaykhiev et al. 2013). Long-term exposition (20 weeks) to cigarette smoke in mice have shown an increase in stem cells features in pancreatic cells, which include among others the RNA polymerase II-associated factor promoter (PASF1) that is found overexpressed in pancreatic tumors (Nimmakayala et al. 2018). Also in a murine model, long-term cigarette smoke exposure in vivo lowered MSCs and HSCs, while increased gene expression related to proliferation, in what is another warning for the use of stem cells from smokers (Siggins et al. 2014). Smoke is capable of induce stem cells in the route to lung cancer development (Lu et al. 2020). Chronic nicotine exposition modulates epigenome through ROS induction and favors stemness in Hk-2 human kidney epithelial cells, during nicotine-related kidney carcinogenesis (Chang and Singh 2019). Cigarette smoke extract (CSE) impairs stem cell development through induction of pro-inflammatory cytokines, affecting MSCs quality in mice (Cyprus et al. 2018). In human ASCs, in vitro exposition to CSE even at lower doses impaired the chondrogenic and osteogenic differentiation albeit adipogenesis was not affected (Wahl et al. 2016). Even in healthy donor's peripheral blood, hematopoietic progenitor cells response to granulocyte colony stimulation factors is reduced in human heavy smokers but is reversed after smoking cessation (Zhen et al. 2020).

Smoking is one of the conditions that should be considered when selecting stem cells donor from different compartments, so as to guarantee that cells quality should not be compromised. Pro-inflammation, epigenetic changes, overexpression of proliferating cells, changes in stemness, and even the development of cancer stem cells could cause a derangement in the potential recipients of those cells.

Alcohol intake have also been reported as detrimental over certain types of stem cells (Dhanabalan et al. 2018), but the literature is more scarce on the subject that reflects the obviousness of avoids selection of alcoholic subjects as cells donors. Neurogenesis is affected by alcohol intake, through depletion of stem cells Sox-2 progenitors (Le Maître et al. 2018). On the opposite side, a moderate red wine intake have been reported to favor the increase of endothelial progenitor cells (EPCs) limited tumor necrosis factor-alpha-induced EPC senescence in what is attributed to an increase in nitric oxide availability (Huang et al. 2010). Resveratrol increases the number of EPCs and angiogenesis (Lu et al. 2019).

Different conditions have shown to be detrimental for stem cell performance, i.e., hyperglycemia (Chen et al. 2007; Yin et al. 2021), that reduce proliferative capacity (Wang et al. 2018). In stem cells where metabolism is high, in concordance with the

increased proliferation requirements, hyperglycemia and O-GlcNAcylation induce genetic instability and trigger DDR in different stem cell types (Na et al. 2020). However, there are differences in stem cell susceptibility to hyperglycemia and ischemia, and one example is the maintenance of genetic stability in MSCs derived from umbilical cord and placenta (Sharma and Bhonde 2015). Human mesenchymal stromal from umbilical cord from newborns from diabetic mothers shows impaired proliferation as well as high levels of p16 of p53, mitochondrial dysfunction, and premature senescence (Kim et al. 2015). In another study in human umbilical cord cells, less proliferation, telomerase function, lower antioxidant enzymes, reduced stemness, less expression in genes related to mitochondrial activity, and impaired differentiation capacity have been observed when pregnancy was affected by gestational diabetes (Kong et al. 2019).

Developing IPSCs from diabetic patient's cells have been proposed to circumvent the problem posed by the heavy mutation burden in islets cells from diabetic donors and open new roads for autologous transplant for diabetic patients (Maxwell and Millman 2021).

Hyperinsulinemia observed in telomerase immortalized MSCs (ASC52telo) is accompanied by a reduction in their capacity to differentiate into adipocytes (Kulebyakin et al. 2021). Insulin resistance-induced oxidative stress have been related with damages that are severe enough so as to consider this relevant metabolic disturbance as a warning condition when evaluating possible stem cells donors (Kulebyakin et al. 2021).

Conclusion

Genome, genetic, or chromosome instability in stem cells from different species are terms that have been in use as synonyms, and those terms describe an increase in sequence variations at molecular level, the presence of aneuploidy, changes in epigenetic patterns, as well as dysregulation of those gene circuits that control DNA damage response and its interconnection with cell cycle control and progression. These changes are the result of a large array of causes operating at different levels, from the oxidative species generated in metabolism or inflammation to those aroused from external challenges to the cell in vivo or during isolation, culture, and reprogramming. Stem cells stemness shows an outstanding dependence from DNA stability and intranuclear deployment and chromatin organization. Those properties that make stem cells the core of the cellular therapy, like stemness, proliferation capacity, and paracrine effectiveness, are linked to genetic stability. Induced pluripotent stem cell reprogramming is paradoxically the "spring of youth" for inducible cells and the source of undesirable mutations on the other side. Consequently, cell dependence on the procedures integrative or not is an issue that will be in the center of stem cell basic and therapeutic-related research.

Genetic stability, of those cells intended for the treatment of many diseases that otherwise would remain without effective therapy, is a must as is the comprehension of those issues regarding to their genetic stability like, isolation, culture conditions

(oxygen levels, type of medium, supplemented, or defined, feeder layers carriers, passage number, handling, and conservation, as well as the adequate selection of donors, even for autologous treatments. The pursue of genetic stability both for standardized cell pools to be commercialized like therapeutic drugs and for individualized medicine will be the consequence of the search for the best combinations between the multifaceted characteristics that will be responsible for the attainable genetically stable low risk therapeutic stem cells.

Cross-References

- ▶ Effects of 3D Cell Culture on the Cell Fate Decisions of Mesenchymal Stromal/Stem Cells
- ▶ Induced Pluripotent Stem Cells
- ▶ Mesenchymal Stem Cell Secretome: A Potential Biopharmaceutical Component to Regenerative Medicine
- ▶ Response of the Bone Marrow Stem Cells and the Microenvironment to Stress
- ▶ The Stem Cell Continuum Model and Implications in Cancer

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