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Phillip George Stubblefield
Editors

Regenerative Medicine

Using Non-Fetal Sources of Stem Cells

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To all scientists – present, past, and future – whose sincere belief in God has made science the true source of religion (philosophy) in their lives.

Dr. Niranjan Bhattacharya, Calcutta, India

Prof. Phillip Stubblefield, Boston, USA

Foreword

Regenerative medicine offers potential treatments and even cures for a range of debilitating diseases such as diabetes, heart failure, and neural disorders. The challenge facing the field is how to isolate cells capable of repairing tissues and organs and to deliver these cells in an effective manner. Stem cells offer a unique starting material for generating cellular products; however, the different sources of stem cells have both advantages and disadvantages. Most likely the optimal source of stem cells will vary for each tissue and application. Embryonic stem cells (ESCs) have the advantage for producing large numbers of cells in vitro; however, these cells have the potential to form tumors and need to be differentiated prior to infusion. Adult stem cells are not associated with tumor formation like ESCs but are difficult to expand in vitro.

The chapters in this book focus on adult stem cells from a range of tissues including abdominal fat, menstrual blood, and dental tissue. Topics deal with the collection, isolation, and characterization of stem cells from these sources. In addition, the therapeutic applications are discussed. Collection of stem cells from these sources is noninvasive and virtually unlimited in supply. These sources offer the potential to generate both autologous and allogeneic stem cell products, and the stem cells can be easily banked for future use. As these tissues are essentially discarded, there are no ethical issues such as those associated with ESCs.

One type of stem cell which has been isolated from a number of tissues and organs is the stromal stem cell which is similar in morphology and phenotype to the bone marrow-derived stromal cell termed mesenchymal stem cell (MSC). The first chapters in this book present an overview of stromal cell-based therapies and the role of stromal cells to support stem cells in their critical role as the microenvironment. Stromal cells (or MSC-like cells) have been isolated from bone marrow, adipose tissue, cardiac tissue, liver, and menstrual blood. These cells secrete a range of growth factors and cytokines that are critical for the proliferation and differentiation of tissue-specific stem cells. The range of factors secreted varies from tissue to tissue and may be a critical component of the differentiation process for individual tissues. One possibility is that the primary effects of treatment with stromal stem cells is through the factors secreted by these cells; however, many studies have also suggested that stromal cells can differentiate into mature cells of various tissues and may directly contribute to generation of new functional cells.

Of course the true test of the potential of stem cells for regenerative medicine is to determine the ability to repair damaged tissue in vivo. This book includes discussion on the results of both animal and human testing of stem cells. Chapters include animal testing in mice and other species. These in vivo studies are essential for identifying the basic mechanisms of actions of stem cells in vivo and determining the extent of integration and differentiation within the damaged tissue. Of course, animal studies are critical also for evaluating the potential of stem cells for tumor development. However, unlike drug testing where the final drug product can be tested in animals, stem cell products from animals vary to their human equivalents. Human-derived cellular products are rejected in animals as xenografts cross species invoke immune rejection. This can be overcome with immunosuppressive drug treatment of the animals; however, this masks potential negative immune responses that could occur. In addition many growth factors and cytokines are species specific and so mouse factors may not stimulate

human cells and minimize potential toxicities. Therefore, carefully designed clinical trials are necessary to fully evaluate cellular products. Several chapters in this book present applications of stem cells in a range of disease settings including stroke and neural disorders.

The editors, Drs. Bhattacharya and Stubblefield, have brought together a comprehensive set of articles from preeminent scientists working on regenerative medicine. The book describes unique sources of stromal stem cells that have great potential for clinical applications and represents a timely addition to the literature in this rapidly changing field. The book will be a resource for both active researches and those entering this field.

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Preface

Evolution of man is a scientific fact. In ancient mythologies, be they from Greece, India, or China, there are stories of kings and emperors seeking the "fountain of youth" or "pearls" that would rejuvenate them so that they can rise above the fundamental problems of life,, i.e., jara, byadhi, and mrityu ((the problem of aging, disease, and death); why is this so? This is the fundamental question of Buddha who left his family and became a monk in search of an answer. The question remains even though science today has evolved from what was once the domain of spirituality, healers, and black magic practitioners. Many healing methods are found in the ancient literatures of traditional Chinese and Indian (Ayurveda) medicine. There are stories of kings and emperors seeking the "fountain of youth" or "pearls" that would rejuvenate them. The so-called Philosopher's Stone that medieval alchemists searched for fruitlessly was supposed to not only turn any substance into gold but also to prolong life and restore youth. Ancient Indian sages practiced "Siddha Vaidya" as well as "trantric" methods for the same reason.

In contemporary times, with a better understanding of the human body down to cellular structures and the DNA along with a better knowledge of debilitating diseases and their impact, scientists are looking not at rejuvenation but regeneration.

A natural effect of aging is degeneration; every organ in a human body degenerates as it ages, leading ultimately to, as they say, death due to old age. Congenital defects and damage can also affect organs like the liver, the heart or the kidney, causing loss of function. Diseases like Parkinsonism or diabetes also cause specific organs to dysfunction. Many of these diseases are also associated with aging, and in today's world, improved healthcare has resulted in increasing longevity. Many significant human diseases arising from the loss or dysfunction of specific cell types in the body, such as Parkinson's disease, diabetes, and cancer, are becoming increasingly common. So far, there has been little reprieve from such debilitating diseases or from damage caused by burns or other accidents. Today, however, a new branch of medicine, regenerative medicine, shows much promise.

The term probably comes from a 1992 paper of Leland Kaiser, "The Future of Multihospital Systems," where in a paragraph subtitled "Regenerative Medicine," the author noted that a "new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems" (Kaiser L. Top Health Care Finance, 1992 Summer; 18:4: 32–45). With work on stem cells getting a new boost in recent years, the process of regenerating dysfunctional and aging organs appears to be no longer a myth but a reality.

Regenerative medicine refers to that branch of medicine which deals with living functional tissues that help to repair or replace damaged or aging tissues, thus regenerating the organ concerned. Research in this field includes cell therapy involving stem cells or progenitor cells, induction of regeneration by biologically active molecules, tissue transplantation, tissue engineering, and the use of cord blood, to mention a few.

Regenerative therapies have been demonstrated (in trials or in the laboratory) to heal broken bones, burns, blindness, deafness, heart damage, nerve damage, etc. It has the potential to cure diseases through repair or replacement of damaged, failing, or aged tissue. Therapies include regeneration of tissues in vitro for future use in vivo as well as direct placement and regeneration

of tissue in vivo. However, this area of medicine is still in its infancy despite the strides made in the last decade. Much of the work is still confined to animal or laboratory models. The next few years are critical as more and more human trials are undertaken and the true potential of this emerging branch of medicine is expressed.

This is the fourth effort by the present book editors to bring together the work of pioneering medical scientists who have ventured into this very exciting field. The first effort resulted in a book, *Frontiers of Cord Blood Science*, which was published by Springer-Verlag in 2009. The focus of the book was on the classical use of stem cells collected from cord blood; other uses of cord blood and its potentials for use in medicine and bioengineering were also emphasized. Our second venture was on the utility of *pregnancy-specific biological substances and its utility in regenerative medicine* (2011). Our third venture was on the utility of aborted fetal tissue in intractable medical diseases (2013). The present fourth book has further broadened the focus to include a variety of non-fetal sources of stem cells and their fascinating role in regenerative medicine. Menstrual blood stem cells, adipose tissue stem cells, stem cells from spongy gum tissue, and even stem cells from breast milk are discussed. Scientists from all over the world have participated in this academic collaboration. This book brings together some of the important work that is being done along with unpublished observations that will help to shape the contours of future therapy in the field of modern regenerative medicine. It promises to be an eye-opener to the enormous potential of hitherto discarded material, like menstrual blood or liposuction material, uprooted decidual teeth, or the operated spongy gum tissue, that had been so far considered as pure biological waste.

It is well known that menstrual blood can rejuvenate a rose tree and its use to attract the lover when you mix it with coffee as per voodoo practice or Baul initiation tradition. The question is what is common in it? Science of botany can support the application of blood or other nitrogenous substances, which can help the growth of the plant in particular. In case of voodoo practice of adding menstrual blood to hot coffee to attract the lover, the stem cell or cytokine niche would be destroyed by the presence of the hot coffee and there would be doubts about the residual pheromone and the residual impact, but still people of that faith are practicing this. Similarly, the menstrual blood of menarche is an important ingredient for the initiation process of the secretive practice of the "Baul" faith. This book has a chapter on this topic. The book will have served its purpose if it acts as a stimulant to professionals and clinical scientists who can build on the knowledge and expand the curative potential of pregnancy-specific biological substances.

Kolkata, India Niranjan Bhattacharya, MBBS, MD, MS, DSc, FACS (USA)

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Dr. Niranjan Bhattacharya and Prof. Phillip Stubblefield

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Introduction

This book is a major contribution to the emerging science of regenerative medicine: using non-fetal sources of stem cells. It is surprising that regeneration often occurs not due to any highly researched and expensive drug; the constituents for regeneration are hidden within the human body itself – it is left to us mortals to discover the potential use of common elements of the human body, since Nature appears to have hidden this science within the human body itself. While conceptualizing this book, the editors wished to put together cutting-edge research on the hitherto near-unknown clinical potentials of simple human substances like fatty tissue collected after bariatric surgery, menstrual blood collected with care and confidentiality, decidual tooth, spongy gum tissue collected from hypertrophic gums, etc. Even breast milk with its hitherto unknown stem cell-like activity is seen to have new disease modifying potentiality on the new born. We firmly believe that the present book will act as a stimulant for senior clinicians and scientists, who may be inspired to further the work of the pioneering scientists who have contributed to this volume. Stem cells and their implications in regenerative medicine are often viewed as a vexed issue because it raises a number of important scientific, ethical, legal, and political questions. These include: ethical concerns regarding ownership, the processes for obtaining consent for collection and storage of human materials, and specific issues relating to confidentiality and privacy, as well as questions raised regarding commercial non-altruistic banking, and social justice issues relating to equity of access and equity of care, just to mention a few. Needless to say, the contributing authors are responsible for their work discussed or delineated in their respective articles; the editors have only helped to bring their pathbreaking ideas and work together, within the covers of this single volume.

Many authors from all over the world have contributed to this pioneering book and the subjects vary from breast milk-derived stem cells and menstrual blood-derived stem cells to the use of adipose tissue and other material in wound healing. Some of the articles are conceptual while others are based on actual experimentation. Maddalena Caruso and Ornella Parolini of the Centro di Ricerca E. Menni, Fondazione Poliambulanza – Istituto Ospedaliero, Brescia, Italy, focus on mesenchymal stem cells, which are at the base of regenerative medicine. Before embarking on an in-depth discussion regarding the concepts of "regeneration" and "repair" using MSC-based therapies, clarification regarding the meaning of these two concepts is paramount. Here, MSC-based therapies for "regeneration" refer to treatments in which MSCs engraft into host tissues and "turn into" (i.e., differentiate into) specific cell type(s) required to replace defective, necrotic, or apoptotic cells and therefore rejuvenate damaged adult tissues; meanwhile, MSC-based therapies for "repair" refer to treatments in which MSCs produce bioactive factors that modulate the local host environment and induce endogenous cells or trigger a cascade of endogenous events, which lead to restoration of damaged adult tissues.

Another contributor is the legendary Professor of Chinese Traditional Medicine, Prof. Leung Ping Chung, Director, Institute of Chinese Medicine, the Chinese University of Hong Kong, Hong Kong SAR. Professor Chung has suggested in his article that animal fats occupy a unique position of utilization in the pharmacopeia of Chinese medicine. Animal fats used in Chinese medicine are those found in the periphery far away from the marrow. Although the animal fats used include a large variety of animals, from domestic to wild types, they share a common indication of "replenishing" for the malnutritioned or debilitated. Another common

use is for skin conditions like various types of injuries and infections. Animal fats in Chinese medicine, therefore, cannot be linked with stem cell regeneration in the modern sense.

Prof. Paul R. Sandberg and his team – Maria Carolina Oliveira Rodrigues, Svetlana Garbuzova-Davis P., Paul R. Sanberg, Júlio C. Voltarellit†, Julie G. Allickson, and Cesario V. Borlongan – from the Center of Excellence for Aging and Brain Repair, University of South Florida, Tampa, Florida, the Ribeirão Preto School of Medicine, University of São Paulo, Brazil, and Cryo-Cell International, Inc., Tampa, FL, have contributed an interesting article on menstrual blood. The team worked on the clinical utility of menstrual stem cells in combating some cardiovascular diseases. Cerebrovascular diseases are the third leading cause of death and the primary cause of long-term disability in the USA. The only approved therapy for stroke is tPA, but its widespread application is severely diminished by the short therapeutic window and hemorrhagic complications, therefore excluding most patients from its benefits. Parkinson's and Huntington's disease are the other two most studied basal ganglia diseases and, as stroke, are plagued with limited treatment options. Inflammation is a key feature in central nervous system disorders and it plays a dual role, either improving injury in early phases or impairing neural survival at later stages. In this chapter the team discussed the role of stem cells as restorative treatments for stroke and other basal ganglia disorders. The recently investigated menstrual blood stem cells are specially emphasized, and their present and future experimental and clinical applications are explored. Professor Zygmunt Pojda, the next author, is a distinguished scientist representing the Department of Cellular Engineering, Maria Sklodowska-Curie Memorial Cancer Center and Department of Regenerative Medicine, Warsaw. He has focused on adipose tissue, which, he notes "contains several types of stem and progenitor cells, including adipose tissue-derived stromal cells (ADSC), endothelial progenitor cells, and the hematopoietic and immune system cells. ADSC share most of phenotypical and functional characteristics of the mesenchymal stromal cell (MSC): bone marrow-derived mesenchymal stromal cell (BM-MSC), or MSC present in cord blood, placenta, and umbilical cord. The basic function of ADSC is the preservation of the adipose tissue integrity by the production of adipocytes in the intensity proportional to their degradation. Recently it has been proven that adipose tissue may contain more MSC-like cells than bone marrow (which serve as the "gold standard" of cells available for autologous cellular therapies. ADSC are able to differentiate not only into adipo-, chondro-, or osteogenic lineages, but also participate in the formation of endothelium, smooth, skeletal or cardiac muscle, hepatocytes, or neural cells."

M. Dhanasekaran, S. Indumathi, J.S. Rajkumar, and R.P. Lissa of the Department of Stem Cells, Lifeline Institute of Regenerative Medicine, Chennai, are a group of researchers from India who are working in the cutting-edge area of human endometrial tissue as a potent source of stem cells. Their chapter provides a brief overview of the current understanding of the evidence supporting the existence of uterine adult stem cells in the endometrial tissue and the role these cells perhaps play during normal adult uterine physiology. It also highlights the advancement of endometrial stem cells in view of its isolation and propagation, its biomarker expression, and its differentiation potential. In addition, it also reviews the possible roles in gynecological disorders associated with abnormal endometrial proliferation and discusses a futuristic approach of applicability of these stem cells for treatment of a wide range of therapeutics. A second contribution by the same team of M. Dhanasekaran et al. reports on the problem of "Subcutaneous Adipose Tissue-Derived Stem Cells: Advancement and Applications in Regenerative Medicine." The investigators reviewed the field and suggested that recent progress in stem cell biology has allowed researchers to investigate distinct stem cell populations in divergent mammalian tissues and organs such as the tendon, periodontal ligament, synovial membrane, lung, liver, endometrial tissue, and body/tissue fluids such as the synovial fluid, the amniotic fluid, and the menstrual blood. Despite the identification of stem cells from various sources, taking those stem cells adaptable for regenerative medicine applications in adequate quantities at the right time is a challenge. In a third contribution by Dr. M. Dhanasekaran et al., they have reported on the problem of omentum fat.

Data reported that identification of putative stem cell population in omental adipose tissue could represent a very useful tool to investigate, at the cellular level, the molecular mechanism and process involved in the onset of obesity and related metabolic dysfunctions. In addition, stromal cells isolated from human omentum fat were identified to retain stem cell characteristics such as proliferative and multilineage differentiation potential inclusive of its angiogenic and regenerative potential. Thus, just as either sides of the coin, omentum fat can be considered both as angels and demons of the body. Increasing the angelic activity and decreasing its demonic activity is of utmost importance for cure of various disorders and its regenerative therapeutics. Humans have enormous quantities of omentum fat as that of subcutaneous fat, and it has been demonstrated from accumulating evidences that the omentum fat can be harvested for the isolation of stem cells, thereby providing omentum fat as a reservoir of stem cells. On the other hand, omentum adipose tissue is more closely associated with an adverse metabolic risk profile than subcutaneous fat.

A very important contribution for this book came from the team of Dr. Graciela Krikun and Dr. Hugh Taylor from Yale University's Dept. Ob/Gyn and Rep. Sci. They have worked on endometrial stem cells as potential cures for human diseases.

According to Drs. Krikun and Taylor, stem cells are undifferentiated cells with the potential to both self-replicate and give rise to other more differentiated cell types. They have tremendous medical potential for use in tissue repair and regeneration and engender optimism for treating some of the most catastrophic illnesses that still lack reliable therapy. Adult stem cells are particularly well suited for immediate therapeutic application; they are immunologically identical to the individual from whom they are obtained and typically do not lead to teratoma formation and do not need genetic transformation. Further, they can often be differentiated using specific culture techniques and defined media without transfection. These characteristics have already allowed the use of adult stem cells in several clinical applications. They have noted in preliminary data that the human endometrium contains multipotent mesenchymal stem cells that can be differentiated into chondrocytes, adipocytes, myocytes, neurons, and pancreatic beta-like cells that secrete insulin.

Another interesting contribution came from Dr. Katarzyna Miernik and Janusz Karasiński of the Department of Cell Biology and Imaging, Jagiellonian University, Krakow, Poland, who have worked on the existence of mesenchymal-like somatic stem cells in the porcine uterus. According to them, "Somatic stem cells are thought to be responsible for remarkable remodeling and regeneration of adult uterus. They are rare, undifferentiated cells able to self-renewal and specialization into all endometrial and myometrial cellular components. So far best characterized by their functional properties, i.e. clonogenicity and ability to differentiate into four lineages (osteogenic, chondrogenic, adipogenic, myogenic) under appropriate in vitro conditions, are mesenchymal stem cells found primary in the human and mouse and recently in porcine uterus. These cells are also defined by the expression of mesenchymal phenotypic markers (e.g. CD73, CD90, CD105) and lack of hematopoietic markers (CD34, CD45). Endometrial mesenchymal stem cells constitute a primary source for menstrual blood stem cells."

Researchers Nurjannah Achmad and Martin Götte of Münster University Hospital, Department of Gynecology and Obstetrics, Germany, worked on the problem of "Characteristics and therapeutic potential of menstrual blood-derived stem cells." According to them, the tremendous regenerative capacity of the human endometrium is based on the activity of adult stem cells. Endometrial stem cells are mainly located in the basal layer but could also be successfully isolated from the functional layer, which is shed during menstruation. Menstrual blood-derived stem cells (MenSCs) can be obtained by noninvasive procedures. They are characterized by high proliferative potential, long-term culturing properties, mesenchymal stem cell-like marker expression, and multilineage differentiation potential. MenSCs have been successfully employed as therapeutics in animal models of myocardial infarction, stroke, Duchenne muscular dystrophy, and critical limb ischemia. Their allogenic application is not associated with immunological side effects and does not promote tumor formation in vivo.

Pilot studies have confirmed their safety upon applications in humans, and phase 1/2 clinical studies on their safety and therapeutic efficacy are ongoing. A systematic banking of immunoprofiled MenSCs will expand the therapeutic repertoire beyond autologous stem cell transplantations.

From Auckland, New Zealand, a very interesting article was contributed by Dr. Michelle B. Locke of the Department of Plastic, Reconstructive, and Hand Surgery, Counties Manukau District Health Board Otahuhu, Auckland, New Zealand, and Dr. Vaughan Feisst of the School of Biological Science, University of Auckland, Auckland. They have written on the problem of "Human Adipose-Derived Stem Cells (ASC): Their Efficacy in Clinical Applications." They are of the opinion that of all of the potential clinical applications for ASC, the most likely to be efficacious is the use of ASC to provide adipose tissue for the reconstruction of soft tissue defects. There is extensive literature on the clinical efficacy of ASC in soft tissue regeneration or reconstruction. Fat injection (FI), where the whole of the aspirated fat is reinjected without any processing to separate or concentrate the ASC, has been performed since 1893 for reconstructive purposes. The 2011 statistics from the American Society of Aesthetic Plastic Surgeons (ASAPS) show that FI are commonly performed by their members, with 69,877 cases reported for the 2011 year. This made it the 9th most common surgical procedure performed, outranking forehead (10th), thigh (14th), and arm lifts (12th) in popularity.

From Vietnam, a state-of-the-art contribution for this book came from the team of Prof. Phuc Van Pham, Drs. Ngoc Bich Vu, Van Ngoc-Le Trinh, Lan Thi Phi, Ngoc Kim Phan, and Phuc Van Pham of the Laboratory of Stem Cell Research and Application, Vietnam National University, Ho Chi Minh City, Vietnam. Their work is on "Human menstrual blood-derived stem cell transplantation for acute hind limb ischemia treatment in mouse models." According to them, "Limb ischemia is a common disease that occurs when there is a sudden lack of blood flow to a limb due to embolism or thrombosis." Currently, treatment of this disease is extremely difficult and shows poor efficacy. De novo angiogenesis based on stem cell therapy is considered as a promising therapy for treating this disease. Therefore, this study aimed to evaluate the efficacy of menstrual blood-derived stem cell (MenSC) transplantation to treat acute limb ischemia in mouse models.

The Vietnamese team contributed a second article on the topic, "Expanded adipose tissue-derived stem cells for articular cartilage injury treatment: a safety and efficacy evaluation." The authors suggested that non-expanded adipose tissue-derived stem cells (ADSCs) are commonly used in preclinical and clinical articular cartilage injury treatment; however, there are usually insufficient non-expanded ADSCs for transplantation. This research aims to evaluate the safety and efficacy of expanded ADSC transplantation in a mouse model. The stromal vascular fraction from abdominal adipose tissue was subcultured for ten passages to enrich for and expand the number of ADSCs. The safety of expanded ADSCs was assessed by evaluating their "stemness" via ADSC-specific marker expression, the expression of two tumorigenesis-related genes (Oct-3/4 and Nanog) using real-time RT-PCR, and in vivo tumor formation in NOD/SCID mice. ADSC efficacy was determined by assessing their chondrocytic differentiative potential in vitro in cartilage-inducing medium, as well as in vivo via injection into a NOD/SCID mice joint failure model. The results showed that expanded ADSCs were negative for Oct-3/4 and Nanog and did not induce tumor formation in mice. Furthermore, ADSCs differentiated into chondrocytes, both in vitro and in vivo, and enhanced the regeneration of articular cartilage in NOD/SCID mice as compared with control. These results confirm that expanded ADSCs are safe and effective for the treatment of injured articular cartilage and offer a promising therapy for degenerative cartilaginous diseases.

From Brazil, Prof. Patricia Pranke and Pedro Chagastelles, Laboratory of Hematology and Stem Cells, Faculty of Pharmacy, Universidade Federal do Rio Grande do Sul Av. Porto Alegre, have written an article on the "Potentialities of adipose-derived mesenchymal stem cells collected from liposuction for use in cellular therapy." They note that the potentialities of ADSCs in medicine are the same for MSCs from bone marrow since they share many of their basic characteristics. Preclinical application of MSCs and ADSCs has been studied for more

than 10 years and the vast majority of the studies show improvements for a wide range of diseases. Some of the mechanisms responsible for the improvements are already known and the principal one seems to be by paracrine secretion of soluble factors, which acts by improving angiogenesis, inhibiting apoptosis, and controlling inflammation in target tissues. A second chapter was contributed by Prof. Pranke with coauthors Luciano Casagrande and Simone Luisi on the topic "Stem cell from dental tissue for regenerative dentistry and medicine." According to them adult stem cells (ASCs) are stem cells found in several different formed tissues, including bone marrow, blood, brain, cord blood, and many other organs, which give rise to different tissues. There are different types of ASCs with different properties. One of the most studied types of ASCs are the mesenchymal stem cells (MSCs). These cells are considered to be an attractive source of cells for regenerative therapies because they have unique plasticity when exposed to different environments. Furthermore, clonogenic cells are capable of self-renewal and differentiation into multiple lines. The first MSCs to be isolated and characterized were from bone marrow (BMMSCs). These cells showed that they have the potential to differentiate into osteoblasts, chondrocytes, adipocytes, and myelosupportive fibrous stroma. MSCs were isolated from various tissues, such as brain, skin, hair follicle, skeletal muscle, and pancreas. In 2000, MSCs were isolated from the pulp tissue of permanent teeth. This discovery opened up a wide range of possibilities for the application of regenerative therapies based on the use of stem cells in oral tissue engineering. So far, five types of human stem cells of dental origin have been isolated and characterized: stem cells from dental pulp (dental pulp stem cells – DPSCs), stem cells from exfoliated deciduous teeth (stem cells from human exfoliated deciduous teeth – SHED), stem cells of the periodontal ligament (periodontal ligament stem cells – PDLSCs), stem cells from apical papilla (stem cells from apical papilla – SCAP), and progenitor cells of dental follicle (dental follicle progenitor cells – DFPCs). These populations of dental stem cells share characteristics common to other populations of mesenchymal stem cells.

Drs. Vladimir Bogin and Thomas E. Ichim have contributed an article on "Endometrial Regenerative Cells and Exosomes Thereof for Treatment of Radiation Exposure." They are from Medistem Inc, San Diego, CA, USA. The authors have commented that in 2007, they discovered a novel subset of mesenchymal stem cells (MSCs) derived from the endometrium, termed endometrial regenerative cells (ERC). In comparison to other MSC types (e.g., bone marrow and adipose), ERC possess (a) more rapid proliferative rate, (b) higher levels of growth factor production (VEGF, GM-CSF, PDGF), and (c) higher angiogenic activity. They are currently running two clinical trials for these cells on patients with critical limb ischemia and heart failure. The main cause of morbidity and mortality in patients suffering from acute radiation syndrome (ARS) is hematopoietic toxicity. Although ARS treatment is not part of routine medicine, commercial interest lies in ability to rapidly obtain FDA approval using the "animal rule," which allows for developers of therapies used in disaster settings circumvention of phase II and III trials if human clinical safety is established and efficacy is demonstrated in a relevant animal model. Recent studies have demonstrated that BM-MSC are capable of preventing lethality subsequent to radiation exposure; however, these cells have performed poorly in late-phase trials. Given that ERC are substantially more economical to manufacture in large numbers and produce more hematopoietically relevant factors as compared to other MSC sources, Bogin and Ichim discuss the possibility of utilizing ERC as a cellular therapy for treatment of radiation exposure.

Prof. Vipin Arora and Dr. Pooja Arora from Haryana, India, have worked on stem cells from decidual teeth. According to the ability and potency to differentiate into different cellular types, three types of stem cells have been established: (1) totipotent stem cells, each cell has the capability of developing into an entire organism; (2) pluripotent stem cells, embryonic stem cells that are grown in vivo under induced conditions and are capable of differentiating into all types of tissue; and (3) multipotent stem cells, postnatal stem cells or adult stem cells with the capability of multilineage differentiation. Dentists are at the forefront of engaging their patients in potentially lifesaving therapies derived from their own stem cells located

either in deciduous or permanent teeth. Postnatal stem cells have been isolated from various dental tissues. The authors mention, like Prof. Pranke et al., five types of dental stem cells have been identified: dental pulp stem cells (DPSC), stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSC), and dental follicle progenitor cells (DFPC). Dental stem cells belong to the multipotent stem cell population. Another interesting contribution in the same field came from Drs. Irina Kerkis1 and Nelson F. Lizier, Laboratory of Genetics, Butantan Institute and Department of Morphology and Genetics, Federal University of Sao Paulo, Brazil. They have noted that it is of common knowledge that differentiation potential of stem cells depends on their anatomic localization as well as on proper niche, among other factors. The perivascular niche of dental stem cells has been described by Shi and Gronthos (2003). The monumental discovery of this group is that this niche is not unique in dental pulp from deciduous teeth and additional niches have been found, which are localized in nerve networks in cell-free zone, in innermost pulp layer in cell-rich zone, and in outermost layer, which contains odontoblast in cell-free zone. The existence of these niches is perfectly consistent with neural crest origin of dental stem cells and isolation of mix population of neuroepithelial and mesenchymal stem cells.

A group of scientists and medical researchers from Australia and Switzerland, Foteini Hassiotou, Donna Geddes, Pilar Blancafort, Luis Filgueira, and Peter E. Hartmann, is a pioneering group who have worked on the stem cell component of breast milk. They have opined that, optimally, breast milk is the only food or drink that infants consume in the first 6 months of life. It is the optimal source of nutrition and protection for the human infant, containing nutritional agents and an array of bioactive factors that confer active immunity to the infant. As breast milk composition is being researched, more and more factors are discovered in it with beneficial attributes. Interestingly, among its biochemical components, breast milk contains maternal cells. Most of our knowledge on breast milk cells comes from studies of leukocytic populations, which often are the dominant cells in colostrum and early lactation milk. However, in mature human milk leukocytes are usually found in low numbers and the dominant cells are of epithelial origin. With the recent technical advancement in the fields of molecular biology and flow cytometry, the epithelial compartment of human milk has started to be further explored. These initial efforts are revealing a profound cellular hierarchy in breast milk, from early-stage stem cells to progenitors to more differentiated lactocytes and myoepithelial cells. The origin(s) of these cell populations and their wide variability among and within women is also starting to be explored. Exciting advances are suggesting the existence of pluripotent stem cell populations in breast milk, and this provides new avenues for examination of the role(s) of these cells in the lactating breast and for the breastfed infant and their potential uses in the study of the biology and pathology of the breast as well as in regenerative medicine.

Professor Colin McGuckin, a global pioneer in stem cell biology from the Institute of Genetic Medicine, Newcastle University, has worked on application of umbilical cord and cord blood as alternative modes for liver therapy. According to him, regenerative medicine has recently shown promise in the management of various human diseases. Recent reports of stem cell plasticity and multipotentiality have raised hopes of stem cell therapy offering exciting therapeutic possibilities for patients with chronic liver disease. With the understanding that stem cells might not just be about making organs ex vivo but also regenerating a patient's own tissues, a concept is now developing to use stem cells to treat patients with serious disease conditions that are terminal or where conventional modes of treatment are insufficient.

Liver cirrhosis and/or liver malignancies have been nominated as the 5th leading cause of death worldwide. The WHO reported, in 2006, that 20 million people around the globe suffer from some form or other of severe liver illness. The ultimate fate of end-stage liver disorders is hepatic dysfunction and eventually organ failure. The only curative mode of management for liver failure is liver transplantation, which is subject to many limitations. Novel alternatives, such as artificial and bioartificial support devices, only aid in temporary replacement of some liver function until an organ is available for transplantation. These newer modalities also have drawbacks or remain experimental and still demand further controlled trials to

allow proof of concept and safety before transferring them to the bedside. There exists a choice of stem cells that have been reported to be capable of self-renewal and differentiation to hepatobiliary cell lineages both in vitro and in vivo. It may, however, be argued that with a global population of 6 billion people and a global birth rate in excess of 130 million per year, the products of birth, umbilical cord and cord blood, possibly provide the most readily accessible and ethically sound alternative source of stem cells. The differentiated stem cells can be potentially exploited for gene therapy, cellular transplant, bioartificial liver-assisted devices, and drug toxicology testing and use as an in vitro model to understand the developmental biology of the liver.

Professor Ian McNiece is a legendary scientist and pioneer of stem cell science. He has contributed a chapter on "The Role of Microenvironment Stromal Cells in Regenerative Medicine." Professor McNiece is currently at the Department of Stem Cell Transplantation and Cellular Therapy, MD Anderson Cancer Center, Houston, Texas. According to him, regenerative medicine offers the potential for treatment and possible cure of debilitating diseases including heart disease, diabetes, Parkinson's disease, and liver failure. Approaches using stem cells from various sources are in preclinical and clinical testing stages. The goal of these studies is to deliver cellular products capable of replacing damaged tissue and/or cells. However, the balance between cellular proliferation and differentiation is a carefully controlled process involving a range of growth factors and cytokines produced in large part by tissue stromal cells. These stromal cells make up the tissue microenvironment and appear to be essential for normal homeostasis. We hypothesize that tissue damage in many instances involves damage to the microenvironment resulting in a lack of signals through growth factor networks necessary to maintain survival and proliferation of tissue-specific stem cells and progenitor cells. Therefore, optimal repair of disease tissue must account for the damage to the stromal environment and will require reconstitution of the microenvironment to support the survival, proliferation, and differentiation of the tissue-specific stem cells or progenitor cells. Further, stromal cells from different tissues have distinct gene profiles, and so a homologous source of stromal cells would minimize potential differences that could result in unwanted toxicities or biological effects.

Quite a few of the articles in this book are contributed by researchers working under the leadership of Dr. Niranjan Bhattacharya at the Calcutta School of Tropical Medicine, India, which is an institute that has hosted the pioneering work on malaria by Nobel Laureate,Sir Ronald Ross and his colleagues. There are also 7 FRS scientists who worked and contributed to knowledge on infectious diseases while working at this institution. Dr. Niranjan Bhattacharya and his associates at the Department of Regenerative Medicine and Translational Science have done some pioneering work in regenerative medicine, some of which is reported in this book. They have also reported their follow-up of many stem cell-based therapies initiated in another government hospital, i.e., Bijoygarh State Hospital, from 1999 onwards, when there were no established guidelines in stem cell research in India. Presently they are following up on many works initiated earlier like: (1) chronic wound of the skin and mesenchymal stem cell-based treatment potential; (2) use of fetal skin and amniotic fluid dressing for nonhealing burn patients in pediatric age group; (3) autologous endometrial tissue transplant, in case of leprosy patients for induction of therapeutic angiogenesis; and (4) uterine synechia, an attempted treatment of the condition with intrauterine instillation of autologous bone marrow mononuclear cells.

There are three joint papers in this book with Prof. Phillip Stubblefield (Editor) and Prof. Sanjukta Bhattacharya of Jadavpur University, and Dr. Niranjan Bhattacharya (Editor) as coauthors on ethics and stem cells, understanding facultative regeneration and its impact on future medicine, and the behavior of Bauls, a secretive sect of Bengal who swallow menstrual blood as a traditional ritualistic practice.The editors express their sincere appreciation to the contributors in this international academic project. Needless to say, the views expressed are those of the individual authors alone and the editors hold no responsibility for the work done by other scientists.

Part I

Basic Science

Multipotent Mesenchymal Stromal Cell-Based Therapies: Regeneration Versus Repair

1

Maddalena Caruso and Ornella Parolini

Introduction

In recent years, a growing wealth of knowledge on the biology and properties of multipotent adult stem cells (ASCs) has resulted in ever-increasing expectations regarding their possible clinical uses, providing new hope for the development of novel and effective cell-based therapies for degenerative diseases, traumatic injuries, and disorders for which there are currently limited therapeutic options.

One of the most extensively studied populations of multipotent ASCs is the mesenchymal stem cells, a population of fibroblast-like, plastic adherent cells which display a defined surface marker profile (CD105, CD73, and CD90 in greater than 95 % of the culture and lack of expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA class II in greater than 95 % of the culture), and is currently termed multipotent mesenchymal stromal cells (with the acronym MSCs), according to the consensus set out by The International Society for Cellular Therapy [1]. Although MSCs were first isolated from the bone marrow (BM), cells which bear MSC characteristics, and which have therefore also been termed MSCs, were subsequently derived from different sites including the adipose tissue [2–4], skeletal muscle [5], liver [6], synovial membrane [7], umbilical cord blood [8], periosteum [9, 10], and peripheral blood [11] and, more recently, from the placental tissue [12], amniotic fluid [13], and menstrual blood [14–16].

However, MSC populations from different origins display some differences in terms of their patterns of gene expression and their differentiation capacity [17, 18]. Such differences might be the consequence of at least two factors. The first of these may be considered a "operational," given that most of the information available on the phenotype and functional properties of MSCs is derived from studies performed on cells cultured in vitro; however, the culture conditions themselves may give rise to the selection of different cell populations and may also induce heritable and epigenetic cellular preconditioning, thereby altering the original cellular phenotype [17–19]. Moreover, comparison among cell populations is made more arduous due to a lack of standardization between isolation and cultivation methods applied in different laboratories [18]. A second factor, which can be considered as an "intrinsic" problem, is related to the in vivo location of MSCs in different tissues, which may differentially influence the commitment, phenotype, and functions of the cells. This aspect is further complicated by the fact that the exact locations of these cells in vivo, as well as their specific natural functions in these locations, are far from being well understood [20]. Finally, a further level of complexity is added to this scenario by the fact that MSCs isolated from specific sites still tend to be heterogeneous populations, which, when cultured, are seen to contain both undifferentiated stem/progenitor cells as well as more mature cell types, which exhibit different functional abilities [18, 20, 21].

Despite these hurdles, much attention has been dedicated to these cells because of their relative ease of isolation, their expansion ability in culture, their multipotency, their absent or low immunogenicity, their immunomodulatory properties, and their ability to home to sites of inflammation or tissue injury (reviewed in [20, 22]). Indeed, all of these characteristics support the notion that MSCs might be valuable candidates for in vivo transplantation and cell-based therapy approaches.

The initial applications for which MSCs have been used in therapy are based on their absent or low immunogenicity and their immunoregulatory functions, as well as their multilineage differentiation capacity. Indeed, on one hand, a major advantage of using human MSCs for in vivo therapies is the fact that these cells are considered to be "immunoprivileged," due to their low expression levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I and their negative expression of major MHC II and co-stimulatory

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molecules such as CD40, CD80, and CD86 (for a review, see [22]). Therefore, allogeneic transplantation of MSCs should not require immunosuppression of the host. In addition, several evidence also show that MSCs may play specific roles in immunomodulation, interacting with cellular components of the immune system and inducing a shift from the production of pro- to anti-inflammatory cytokines [22].

On the other hand, the fact that isolated and expanded MSCs, mostly BM-derived, have been shown to be capable of differentiating into multiple cell types in vitro suggests that these cells might also be useful in a clinical setting for tissue regeneration, with tissue engineering and regenerative purposes [23].

Interestingly, increasing evidence has recently highlighted that MSCs produce bioactive molecules (such as cytokines and growth factors), which are able to exert several types of paracrine effects (e.g., anti-scarring, anti-apoptotic, anti-inflammatory) on target cells [20]. These findings have further widened the scope of possible MSC-based therapeutic applications and forced the reinterpretation of previous results which have been obtained with these cells. Indeed, in vivo studies have revealed that although MSC transplantation improves tissue conditions in several experimental animal models of disease, as well as in human clinical trials, in many cases, such as for the treatment of myocardial infarction or fibrosis (and other cases to be described later in this chapter), the number of engrafted cells and the levels of tissue-specific differentiation of these cells within injured or diseased host tissues are often very low or undetectable and likely insufficient to account for the observed functional improvements. Therefore, in such cases, it seems that cell replacement mechanisms represent only a minor facet of the role of MSCs in tissue regeneration. Conversely, it is becoming increasingly plausible that many of the beneficial effects exerted by MSCs in vivo are related to the bioactive molecules secreted by these cells and to the reparative actions of these molecules, which act by paracrine mechanisms on surrounding host tissues.

Before embarking on an in-depth discussion regarding the concepts of "regeneration" and "repair" using MSC-based therapies, clarification regarding the meaning of these two concepts is paramount. Here, MSC-based therapies for "regeneration" refers to treatments in which MSCs engraft into host tissues and "turn into" (i.e., differentiate into) specific cell type(s) required to replace defective, necrotic, or apoptotic cells and therefore rejuvenate damaged adult tissues; meanwhile, MSC-based therapies for "repair" refers to treatments in which MSCs produce bioactive factors that modulate the local host environment and induce endogenous cells or trigger a cascade of endogenous events, which lead to restoration of damaged adult tissues.

In this chapter, we will focus on those cases in which improvements in tissue function observed after MSC-based therapies do not seem to be related to the "regenerative" capacity of donor cells but, rather, are most likely due to the actions of these cells on the site of injury, thereby constituting a "reparative" activity which is associated with the regeneration of host cells. We will also discuss the concept that "regeneration" and "repair" are not mutually exclusive. Finally, we will show that reparative actions can be exerted at various levels and that identification of the mechanisms underlying the ability of MSCs to induce tissue recovery is becoming an important and challenging area of investigation, which is opening a new chapter in the therapeutic use of MSCs.

MSC-Based Therapy for Skeletal Diseases

MSCs are mainly defined simply in terms of their in vitro ability to differentiate toward the three classical mesodermal lineages (osteogenic, adipogenic, and chondrogenic) under appropriate culture conditions [18, 24–27]. On the basis of these in vitro characteristics, many attempts have been made to exploit the differentiation capabilities of MSCs in vivo to develop MSC-based approaches for the treatment of disorders affecting skeletal tissues and associated connective tissues (cartilage, tendon, and ligament) [28, 29]. Preliminary studies carried out in animal models, followed by preclinical studies and human clinical trials, have provided several evidences to support the feasibility of using MSC transplantation for this purpose, without resulting in the initiation of an immunological response (reviewed in [28]). This strategy is centered mainly on the ability of MSCs to differentiate and "turn into" cells of the specific injured tissue to be restored (regenerative approach), a process which is induced either by transplanting the cells alone or in combination with scaffolds (synthetic or natural and biodegradable), which provide mechanical and structural support, or exogenous factors (growth factors, soluble cytokines, chondrogenic, osteogenic factors), which enhance differentiation of MSC toward cells of the required tissue [28, 30]. For instance, successful results have been obtained in patients for the treatment of bone defects such as long bone nonunion fractures [31] and large bone diaphysis defects [32]. These patients have been treated with ex vivo-expanded autologous BM-derived MSCs encased in porous hydroxyapatite ceramic scaffolds, designed to match the bone deficit in terms of size and shape, and in both cases, treatment has resulted in the integration of the graft and healing of bone defects. A similar approach has also been applied with success in humans for the treatment of spinal fractures/vertebral disk injuries [33] and craniofacial defects [34, 35]. MSC-based therapy has also been suggested for the treatment of cartilaginous injuries. Indeed, numerous studies in both animal models and in humans have reported that transplantation of MSCs in combination with scaffolds results in new cartilage formation [36–38]. For instance,

Wakitani and colleagues [36] have reported treatment of patellar cartilage defects by this approach, whereby histological analyses revealed the successful repair of defects with fibrocartilaginous tissue.

Besides these approaches, which are based mainly on the local injection and implantation of MSCs, systemic transplantation of MSCs has also been shown to be a viable approach for the treatment of bone diseases such as osteogenesis imperfecta (OI). OI is a genetic disorder of bone and other tissues caused by a mutation in the genes coding for type 1 collagen, the major structural protein in bone. This disease is characterized by the occurrence of fractures, reduced bone growth, and progressive bone deformation. Horwitz and colleagues [39–41] were the first to investigate whether MSC transplantation could be used to treat patients affected by OI. In 2002, these authors reported that when children with OI were treated first with a standard allogeneic BM transplant and then with a "booster" of MSCs from the same donor (18 months post BM transplantation), clinical conditions were ameliorated, and the children began to grow again. These authors claimed that donor MSCs can engraft after transplantation and differentiate to osteoblasts as well as skin fibroblasts, thereby conferring clinical benefits attributable to the engraftment of functional mesenchymal precursors.

Even though these findings support the notion that the beneficial effects observed are likely due to the differentiation of MSCs into the cell types needed for tissue regeneration, they still leave open the possibility that differentiation is not the only mechanism underlying the effects observed. For instance, although MSC-based therapy for cartilage regeneration was first conceived on the basis of the ability of these cells to differentiate toward the chondrogenic lineage, in some cases, doubts have been raised concerning the origin of newly formed cartilage [28, 38] and the mechanisms involved [28]. Indeed, it remains to be demonstrated as to whether new cartilage tissue is derived directly from the differentiation of transplanted MSCs, therefore representing a regenerative action of these cells, or from the ability of the transplanted cells, through paracrine mechanisms, to inhibit host inflammatory responses or stimulate the growth and/or activity of endogenous progenitors and chondrocytes [28], and therefore acting through a reparative mechanism. For instance, in the case of diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), which are degenerative joint diseases associated with progressive and often severe inflammation, it seems that the beneficial effects of MSCs are attributable mainly to the induction of endogenous progenitor cells and their anti-inflammatory and immunosuppressive activities [42]. In addition, as is also the case when MSCs have been applied for OI treatment, some authors have reported that the levels of donor MSCs in bone, skin, and other tissues were less than 1 %. Although Horwitz and colleagues [41] claimed that these low levels of engraftment were adequate to confer clinical benefits, a reinterpretation of these results by others questioned whether the beneficial effects observed were due only to MSC differentiation into osteoblasts to form bone and suggested that most probably, bioactive factors secreted by the MSC also supported the observed growth and improvement of clinical conditions [19, 43].

MSCs-Based Therapy for Other Pathological Conditions

Besides differentiation toward mesodermal lineages, efforts have been devoted to investigate the ability of MSCs to differentiate across germinal boundaries outside of the mesenchymal lineage and to therefore also include the endodermal and ectodermal lineages, a process often referred as "trans-differentiation." Although results concerning this property of MSCs are still debated [17, 44], several studies indicate the presence of multipotent cells with MSC characteristics, which, under particular conditions, not only differentiate into cells of the mesodermal lineage but also into cells resembling neurons [17, 45–47], hepatocytes [48–52], and cardiomyocytes [53, 54], as well as endothelial [55] and pancreatic cells [56, 57].

Over the years, a wide variety of experimental conditions have been set up in an attempt to trigger and study trans-differentiation in vitro [17, 52]. Generally, these protocols are based on induction of differentiation by the addition of soluble factors to the culture medium (e.g., growth factors, cytokines, corticosteroids, hormones, chemical demethylating agents), as well as the reconstitution of cell-matrix and cell-cell interactions, with the intent of creating a microenvironment and signals to drive cell differentiation toward a specific lineage in vivo under the normal developmental/homeostatic conditions of a specific tissue/organ [17, 52, 58]. After induction, the potential resulting differentiation is monitored by evaluating cellular morphological changes (i.e., changes to neuron-like, hepatocyte-like, and cardiomyocyte-like features), the expression of various tissue-specific genes, as well as assessing any acquired abilities of the cells to exert tissue-specific functions. The literature currently includes a multitude of papers reporting successful results in this field. Nevertheless, concerns remain regarding the interpretation of the results achieved, given that there is a lack of standardization between the existing reports in terms of the methods used to induce differentiation, as well as in the criteria applied for phenotyping in vitro-generated differentiated cells. Indeed, on one hand, differentiation strategies are hampered by great variability between protocols used by different groups and also by the fact that in most cases, the signals that drive natural differentiation in vivo remain to be completely defined, therefore making it very difficult to reproduce them in vitro. On the other hand, phenotyping is

compromised by several aspects such as (*i*) the lack of specificity of differentiation markers used to evaluate the grade of differentiation achieved; (*ii*) molecular and functional heterogeneity of the starting MSC populations used, which constitutes an additional variable for consideration in efforts to ascertain the transdifferentiation ability of cells; and finally (*iii*) possible artifacts resulting from the fact that the cells used have been removed from their natural in vivo location and are subsequently grown in a nonphysiological, chemical ex vivo environment and may therefore undergo cytoskeletal and phenotypic alterations that might be misinterpreted as a "true" transdifferentiation phenomena [17, 59]. For example, several studies have described methods to direct MSCs to differentiate into specific neuronal subtypes [46, 47]; however, the positivity of the results obtained has been questioned, given that undifferentiated MSCs express a considerable repertoire of neural genes, and therefore, the expression of these genes after induction may not be the result of differentiation (reviewed in [17]). In addition, some of the neural markers, which have been analyzed, such as nestin, are not restricted to neural tissues but are also expressed in a variety of mesodermal cell types [17, 60, 61]. Similar criticisms can be applied to the interpretation of MSC transdifferentiation toward the hepatogenic lineage in vitro. Indeed, the hepatic differentiation markers often employed (such as tyrosine aminotransferase, phosphoenolpyruvate carboxykinase, and liver-enriched transcription factors) are not "true" hepatocyte markers, given that they are also expressed in other somatic cells such as cells of the lung, intestine, pancreas, and kidney or are expressed by MSCs even before induction of differentiation [52, 59, 61–64].

In spite of such limitations, in vitro transdifferentiation of MSCs has been demonstrated repeatedly, and such studies have driven scientists to investigate the potential of MSCs to "transdifferentiate" in vivo after transplantation in animal models. For instance, Kopen and co-workers [65] were one of the first groups to demonstrate that MSCs isolated from BM, when injected into the central nervous systems (CNS) of newborn mice, were able to migrate throughout the forebrain and cerebellum without causing disruption to the host brain architecture. Some of these cells were shown to differentiate into astrocytes, as well as engrafting into neuron-rich regions, suggesting that neural differentiation had occurred. These results were subsequently confirmed by other groups in vivo [44, 66–68]. Similarly, transplantation of MSCs derived mostly from BM, and adipose tissue has been shown to result in engraftment in the heart and differentiation toward the cardiomyogenic lineage [69, 70], while it has been shown that MSCs isolated from different sources may also generate hepatocyte-like cells in vivo (reviewed in [59]).

Despite these promising studies, doubts have again been raised regarding interpretation of the positive differentiation results reported. Indeed, the level of engraftment observed in these studies is generally very low, and the differentiation achieved in vivo has not given rise to fully mature cells and is often poorly characterized. These limitations are likely due to problems with the cell delivery strategies adopted in these studies (i.e., local injection vs. intravenous/systemic administration), as well as to the often questionable analysis undertaken on the phenotype of the differentiated cells, and finally, to the fact that current tracking techniques for the study of engraftment and differentiation remain modest. Furthermore, some researchers suggest that the morphological and phenotypic changes observed in MSCs after transplantation are a result of fusion between donor cells and host cells, rather than true transdifferentiation [71–73].

Even so, MSC-based therapeutic approaches have been tested in a range of animal models of human diseases (followed also by testing in humans), for treatment of conditions such as myocardial infarction, brain and spinal cord traumatic injury, stroke, and fibrosis.

For instance, much effort has been dedicated to investigating whether MSC-based therapy may be beneficial for the treatment of myocardial infarction/ischemia and heart failure. Myocardial ischemia, whether acute or chronic, triggers a cascade of events leading to cellular injury or death, resulting in the sending of signals that cause the inflammatory phase which is characterized by macrophage and neutrophil infiltration, subsequently leading to scar formation, loss of structural integrity and cardiac mass, and ultimately ending, in severe cases, in congestive heart failure [70, 74]. Under these conditions, self-regeneration capacity is extremely limited [58, 70]. A large number of studies have been performed to test the feasibility of MSC-based treatments of such disorders, with the main aim of developing a "regeneration approach": i.e., whereby transplanted MSCs would engraft into host tissues and differentiate into new cells with cardiomyocyte-like features and functions, thereby correcting the heart failure through the replacement of dead resident cells. By the time the studies to test this hypothesis were conducted, MSCs were indeed thought to contribute to tissue function by means of differentiation and replacement mechanisms. Transplantation of MSCs into post-infarct animal models was shown to improve post-ischemic cardiac functions and trigger a reduction in infarct size and, in some cases, to decrease mortality [75–79]. However, one of the most intriguing observations was that the transplanted cells frequently produced functional improvement despite the small numbers of cells which were seen to be engrafted in recipient heart tissues. Furthermore, in many studies, the transplanted cells did not persist in the recipient animals in the long term, while in other reports, this factor was not even investigated. Meanwhile, some authors showed that the in situ differentiation of transplanted MSCs in the heart toward the

cardiomyocyte lineage was often incomplete, while in other cases, this was only partially characterized or not assessed at all [58, 70]. Iso and colleagues [80] reported a significant improvement in cardiac function and fibrosis after infusion of human MSCs into immunodeficient mice with acute myocardial infarction, despite the fact that no engrafted donor cells could be detected after 3 weeks postinjection. Furthermore, Dai et al. [81] found that allogeneic MSC transplantation into a rat myocardial infarction model resulted in an improvement of global left ventricular function at 4 weeks and that donor cells survived in the infarcted myocardium for up to 6 months, with expression of markers that suggest that the transplanted cells had differentiated toward muscle and endothelial phenotypes, although without fully adopting an adult cardiac phenotype, and not resulting in a visible replacement of scar tissue with sheets of muscle cells. Intriguingly, the time needed for the MSCs to differentiate toward the myogenic lineage was longer than expected, while the therapeutic effects of the injected MSCs were evident even before cells expressing cardiac-specific markers could be detected and within a time frame that was too short to reflect the occurrence of true regeneration. Therefore, the mechanisms whereby transplantation of MSCs improved cardiac function remained to be further investigated, but the authors suggested that a transient paracrine mechanism may have been at play. Strong support for paracrine actions of MSCs in cardiac repair have come from studies performed by Gnecchi and colleagues [82, 83]. In particular, these authors demonstrated that the administration of conditioned (and therefore cell-free) medium from MSCs overexpressing Akt-1 (a prosurvival gene) in a rat model of coronary occlusion resulted in a reduction in infarct size and cardiac apoptosis, possibly through the release of paracrine factors, such as VEGF (vascular endothelial growth factor), FGF-2 (fibroblast growth factor-2), HGF (hepatocyte growth factor), IGF-I (insulin-like growth factor-1), and TB4 (thymosin ß4) [83]. Since some of these factors could also have proangiogenic activities, their paracrine functions may have been responsible for inducing neovascularization in the injured heart [83, 84].

Our group has also recently hypothesized paracrine mechanisms to explain the observation that application of amniotic membrane fragments (known to also contain MSCs) onto infarcted rat hearts significantly reduces post-ischemic cardiac dimensional alterations and improves myocardial function for up to at least 60 days after ischemia [85]. Interestingly, in this study, no engraftment of amniotic cells was detected in host cardiac tissues, suggesting that the benefits observed may not have been related to engraftment of amniotic cells into the ischemic rat hearts, but more likely, due to release of soluble factors that may have modulated the ischemic inflammatory process, resulting in prolonged survival of host tissue cells.

However, it is important to keep in mind that "regeneration" and "repair" do not necessarily mutually exclude each other. For instance, Amado and colleagues [76] claimed that the cardiac improvements exerted by BM-derived MSCs in pigs with myocardial infarction might be the result of both mechanisms: transdifferentiation of transplanted MSCs toward the cardiomyocyte lineage (regeneration) and increased endogenous reparative mechanisms (repair), potentially through the release of factors such as VEGF, which is linked to both neoangiogenesis [86] and stem cell homing and migration [87].

MSC-based therapy has also been investigated for the treatment of several models of CNS diseases, affecting both the brain [traumatic brain injury and cerebral infarct (ischemic stroke)] and the spinal cord (traumatic spinal cord injury) [88]. Although it has been suggested, as reported above, that differentiation of MSCs into cells of neural lineage may occur both in vitro and in vivo, in most of these studies, regeneration through MSC transdifferentiation is, once again, unlikely to be the major mechanism underlying the observed functional recovery. Indeed, these studies reported that in general, few of the transplanted MSCs expressed astrocytic or neural markers, and these were far too few in number to provide cellular replacement. In particular, Mahmood and colleagues [89] found that MSC transplantation into a rat model of traumatic brain injury resulted in increased endogenous cell proliferation and improved functional recovery, with only few MSCs observed to express neural markers. Conversely, in more than one case, functional improvement was observed in association with an increase (either locally or in the cerebrospinal fluid) in the levels of soluble factors, such as the neurotrophic factors NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor) [90, 91], GDNF (glial cell line-derived neurotrophic factor), activin A, TGFβ-1 (transforming growth factor-1), and TGFβ-2 [92]. Similarly, intravenous administration of MSCs into a rat model of stroke was shown to improve functional recovery, increase FGF-2 expression, reduce apoptosis, and promote endogenous cellular proliferation [93]. Although results regarding the use of MSCs for the treatment of spinal cord injury remain controversial, many studies have provided evidence that administration of these cells may also induce functional recovery in this scenario, even when only a low level of neural differentiation is documented [88].

MSC-based approaches have also been explored for liver disorders. Although much effort has been dedicated to testing whether cell therapy using MSCs could be used as a potential alternative to hepatocyte transplantation in order to cure metabolic and acute liver diseases, with debatable results obtained to date [59, 94], other authors have been prompted to investigate whether MSCs, mainly BM derived, could be used for the treatment of chronic liver disorders such as liver fibrosis. The results which have been obtained so far are controversial (i.e., reduction versus enhancement of fibrosis),

and open questions also remain regarding the mechanisms involved [95, 96]; however, at least two possible mechanisms have been proposed for the potential therapeutic function on liver fibrosis exerted by MSCs: one implies their ability to engraft into the liver and differentiate toward the hepatogenic lineage, therefore participating in the regeneration of the endogenous parenchyma; the other possible mechanism is related to the ability of MSCs to produce or activate paracrine mediators, such as IL-10 (interleukin-10), TNF-α (tumor necrosis factor-α), and HGF, which lead to reduction/modulation of fibrosis (for a review, see [96] and [97]). For instance, Parekkadan et al. [98] have shown in vitro that MSCs produce IL-10 and TNF-α, which may have inhibitory effects on proliferation of hepatic stellate cells (HSC) (one of the main sources of ECM-producing myofibroblasts) and collagen synthesis, while MSC-derived HGF was seen to be responsible for a marked induction of HSC apoptosis. In addition, Chang et al. [99] reported that after the injection of human BM-derived MSCs labeled with GFP into a rat model of liver fibrosis [carbon tetrachloride (CCl4) induced], liver fibrosis was significantly decreased, and the degree of fibrosis reduction paralleled the number of donor cells observed in liver sections. Although these authors reported dubious results regarding differentiation of MSCs into hepatocytes, they also suggested that the observed decrease in fibrosis could have been due to production by MSCs of matrix metalloproteinases (MMP) with anti-scarring activity, as well as HGF, which could have exerted anti-apoptotic effects, thereby increasing hepatocyte proliferation. Paracrine effects have also been hypothesized by Tsai et al. [100] to explain an observed reduction in liver fibrosis in the absence of differentiation of engrafted Wharton's jelly-derived cells, which had been transplanted into rats with (CCl4)-induced liver fibrosis, probably by inducing a reduction in the expression of profibrogenic TGF-β1 by biliary epithelial cells.

MSC-Based Therapy for Immune-Related Diseases

Numerous studies have demonstrated that MSCs are able to modulate the function of different immune cells in vitro, in particular T lymphocytes and antigen-presenting dendritic cells (DCs), which play a key role in the induction of immunity and tolerance. Indeed, MSCs are able to suppress T lymphocyte activation and proliferation in vitro. This inhibition affects the proliferation of T cells after stimulation by alloantigens [101–103], mitogens [104], as well as activation of T cells by CD3 and CD28 antibodies [103, 105]. Most studies in this regard have reported that MSCs exert their suppressive function by means of soluble factors such as TGF-β and HGF [104], prostaglandin E2 (PGE2) [103], and the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) [106]. MSCs may also modulate immune responses through the induction of regulatory T cells [107]. Moreover, MSCs have been demonstrated to interfere with differentiation, maturation, and function of antigen-presenting DCs, likely by means of soluble factors such as IL-6 (interleukin-6) and PGE2 [108–110]. Furthermore, MSCs may also modulate B-cell functions [111, 112] and affect the cytotoxic activity of natural killer (NK) cells by the inhibition of proliferation and cytokine secretion [110, 113].

Intriguingly, several studies suggest that MSCs may exert their immunoregulatory functions specifically at sites of inflammation. Indeed, it has been shown that when MSCs are delivered intravenously in animal models, they home preferentially to sites of inflammation, where they respond to signals from the surrounding microenvironment and perform local immunoregulatory actions (reviewed in [114]). This "homing" ability has been attributed to the expression of receptors for growth factors, chemokines, and extracellular matrix on the surface of MSCs, which mediate the migration of these cells to the injured site [115, 116].

The immunosuppressive properties of MSCs have been examined in a variety of animal models, as well as in clinical studies. Although the mechanisms involved are only partially known and still under study, they very likely involve both contact-dependent mechanisms and production of soluble factors. To date, MSC-based approaches have been investigated for the treatment of alloreactive immunity (to reduce or prevent graft rejection after cell and organ transplantation), autoimmunity (to ameliorate experimental autoimmune conditions, such as multiple sclerosis (MS) and Crohn's disease), and also tumor immunity.

In particular, significant studies in this field have been performed for the treatment of the graft-versus-host disease (GVHD), a life-threatening complication arising after allogeneic BM transplantation. In this condition, cells of the immune system, which are present in allogeneic donor BM, recognize the recipient's cells as foreign and attack them, with a high risk of mortality. Interestingly, Le Blanc and co-workers [117] demonstrated that the infusion of haploidentical MSCs into a patient with severe GVHD of the gut and liver resulted in rapid recovery from acute GVHD in the gastrointestinal tract and the liver. Furthermore, other clinical studies have also applied MSC-based treatments for patients with steroid-resistant, severe acute GVHD in a multicenter, phase II experimental study [118], whereby transplantation was performed using MSCs derived from the European Group for Blood and Marrow Transplantation's ex vivo expansion procedure. More than half of the enrolled patients with steroid-refractory acute GVHD responded to treatment with MSCs, and no patients showed any side effects either during or immediately after infusions of MSCs. Two years later, just over half of those patients with a complete response were still alive. Despite these promising results, little is known about the mechanisms

exploited by MSCs to induce such beneficial effects, partly due to the fact that few data are available concerning cell survival after transplantation. Indeed, most data derived from animals indicate short survival of MSCs after injection in vivo. Le Blanc and colleagues [117] suggested that the observed clinical benefits might not require sustained engraftment of many cells but could instead result from production of growth factors or temporary immunosuppression.

MSC-based approaches have also been tested with success in rodent models of diseases such as MS and diabetes, where immunomodulation is thought to be the main operative mechanism [119, 120]. For instance, transplantation of MSCs has been shown to ameliorate the conditions of mice affected by experimental autoimmune encephalomyelitis (EAE), a murine model of human MS, which is a chronic inflammatory multifocal demyelinating disease of the CNS that predominantly affects young adults [121–123]. Zappia and colleagues [121] demonstrated that injection of MSCs in EAE mice significantly reduced the clinical severity of EAE, with a decrease in CNS inflammation (suppression of effector T cells and induction of peripheral tolerance, decreased infiltration of the CNS by T cells, B cells, and macrophages), induction of T-cell anergy at the level of lymphoid organs where MSCs seemed to engraft, and reduction of demyelination both in the brain and spinal cord of treated mice. Recently, an MSC-based therapy (intrathecal injection of autologous MSCs) has been investigated for treating patients affected by MS based on the notion that MSCs can migrate locally into the areas of lesions, where they have the potential to support local neurogenesis and rebuilding of the affected myelin [124, 125].

MSCs are also very attractive candidates for the treatment of the amyotrophic lateral sclerosis (ALS), which represents another devastating and incurable neurodegenerative disease targeting motor neurons and their connections to muscle. Human MSC transplantation has been shown to extend survival, improve motor performance, and decrease neuroinflammation in the SOD1(G93A) mouse, a murine model of ALS [126]. MSC transplantation has also been tested in patients with ALS in two phase I clinical trials, demonstrating that this procedure was safe and well tolerated and might be applicable in future cell-based clinical trials for ALS; however, the lack of postmortem data prevents any definitive conclusions regarding the presence of the MSCs after transplantation to be drawn [127].

Potential Paracrine Effects of MSCs and the Biologically Active Molecules Involved

At this point in our discussion, it is perhaps worth summarizing the putative paracrine effects of MSCs and the corresponding molecules involved in these effects. To this end, we can refer to classification of the group of Caplan [20, 128] which proposes that the paracrine effects of MSCs should be divided into (*i*) anti-apoptotic effects, through reduction/inhibition of apoptosis of resident cells, therefore limiting the area of injury; (*ii*) anti-fibrotic and anti-scarring effects, by suppression of the inflammatory response, modulating protease activity, and production of extracellular matrix; (*iii*) angiogenic effects, through promotion of angiogenesis and restoration of blood flow around the damaged area; (*iv*) supportive effects, by stimulating proliferation and differentiation of endogenous stem/progenitor cells; and, finally, (*v*) immunomodulatory effects, through inhibition of the proliferation of CD8+ and CD4+ T lymphocytes and NK cells, suppression of immunoglobulin production by plasma cells, inhibition of maturation of DCs, and stimulation of regulatory T-cell proliferation.

Table 1.1 reports some of the molecules involved in these processes and relative examples, even though the roles of many of the bioactive molecules implicated remain to be validated. These molecules may have pleiotropic effects, and their collocation in one of the abovementioned groups of paracrine effects rather than another is not restrictive. Moreover, it has recently been proposed that, besides soluble factors, cell-derived microvesicles, consisting of proteins and lipids that may also contain nucleic acids (mRNA, miRNA, and DNA), might also represent a new mechanism of cell-to-cell communication through which paracrine effects may be exerted, with the transfer of signals and molecules from one cell to another even over long distances [143, 144].

Although classification of paracrine effects and molecules could help in our understanding of this complex area of investigation, we are still far from understanding all of the mechanisms and molecules involved. Moreover, it is important to underline the fact that the molecules and mechanisms proposed should not be seen as separate actors in the paracrine actions exerted by MSCs, but rather, these actions should be viewed as the results of a combination of factors and mechanisms that work in concert to modulate the molecular composition of the local tissue environment to evoke responses from resident cells.

MSC-Based Therapy and Clinical Trials

Interestingly, in the last decade, the number of clinical trials using MSCs to treat a wide range of damaged, diseased, or inflamed tissues has been rapidly increasing. Indeed, a quick search of the site www.clinicaltrials.gov using “mesenchymal stem cells” or “mesenchymal stromal cells” as a search query and selecting only “interventional studies” (studies where individuals are assigned to receive specific interventions) and returns more than 400 trials, all of which are aimed at

Table 1.1 Potential paracrine effects of MSCs and the biological active molecules involved

Paracrine effect	Molecule	Properties	Examples
Anti-apoptotic	VEGF	Member of the platelet-derived growth factor family	MSC-secreted VEGF decreases apoptosis of endothelial and tubular cells [129]
			Adipose tissue-derived MSCs secrete VEGF and prevents cardiomyocyte apoptosis [130]
	HGF	Multifunctional factor: mitogenic, motogenic, morphogenic, and anti-apoptotic	MSC-secreted HGF decreases apoptosis of endothelial and tubular cells [129]
			Adipose tissue-derived MSCs secrete HGF [131]
			MSC-secreted HGF may decrease apoptosis and increase hepatocyte proliferation [99]
	IGF-1	Insulin-like hormone	Adipose tissue-derived MSCs secrete IGF-1 and prevent cardiomyocyte apoptosis [130, 131]
Anti-fibrotic and anti-scarring	HGF	Multifunctional factor	HGF antagonizes the pro-fibrotic actions of TGF-β by intercepting Smad signal transduction [132]
			Adipose tissue-derived MSCs secrete HGF contributing to suppression of fibrogenesis [133]
	IL-10	Cytokine	MSC-secreted IL-10 may inhibit HSC proliferation and collagen synthesis [98]
	TNF-α	Cytokine	MSC-secreted TNF-α may modulate HSC proliferation and collagen synthesis [98]
	MMP	Zn(++)-endopeptidases able to degrade ECM	BM-MSCs express MMP resulting in a significant reduction in liver fibrosis [99]
Supportive	HGF	Multifunctional factor	MSCs-secreted HGF stimulates proliferation of surviving cells [129]
	LIF	Interleukin-6 class cytokine	BM-derived MSCs express LIF and support hematopoiesis in vitro [134]
	SCF	Cytokine that binds c-Kit	BM-derived MSCs express SCF and support hematopoiesis in vitro [134]
	IL-6	Cytokine	BM-derived MSCs express IL-6 and support hematopoiesis in vitro [134]
	M-CSF	Cytokine	BM-derived MSCs express M-CSF and support hematopoiesis in vitro [134]
	FGF-2	Member of the fibroblast growth factor family	MSCs increase FGF-2 expression and promote endogenous cellular proliferation after stroke [93]
Angiogenic	FGF-2	Member of the fibroblast growth factor family	FGF-2 promotes angiogenesis directly or indirectly, by upregulating VEGF [135]
			MSC-secreted FGF-2 enhances proliferation of endothelial and smooth muscle cells [136]
	VEGF	Member of the platelet-derived growth factor family	MSC-secreted VEGF enhances proliferation of endothelial cells [136]
			MSC transplantation induces VEGF and neovascularization in ischemic myocardium [137]
	MCP-1	Small cytokine of the CC chemokine family	MSCs secrete MCP-1 [138]
			Chemoattractant protein that helps the migration of endogenous stem cells to injured sites [135]
	IL-6	Cytokine	MSCs secrete IL-6 [138]
			IL-6 induces the expression of VEGF [139]
	Angiogenin	Heparin binding protein of the RNAse superfamily	Induces new blood vessel formation [140]
			Conditioned medium from MSCs contains angiogenin [138]
	PIGF	Member of the VEGF subfamily	PIGF promotes prenatal and postnatal angiogenesis [135]
			BM-MSCs secrete PIGF [136]
Immunomodulatory	PGE-2	Lipid compound of the prostanoid class of fatty acid derivatives	MSCs constitutively produce PGE2 [103]
			PGE-2 modulates the MSC effects on T cells and NK and DCs [110, 141]
	TGF-β	Cytokine	Mediator for suppression by MSCs of T-cell proliferation [104]
			Responsible for MSC-mediated inhibition of NK proliferation [141]
	HGF	Multifunctional factor	HGF mediates antiproliferative effects of MSCs on T cells [104]
	IDO	Immunomodulatory enzyme	IDO mediates suppression of T-cell proliferation by MSCs [106]
	iNOS	Member of nitric oxide synthases family	iNOS mediates suppression of T-cell proliferation by MSCs [142]

Abbreviations: *VEGF* vascular endothelial growth factor, *HGF* hepatocyte growth factor, *IGF-1* insulin-like growth factor-1, *IL-10* interleukin-10, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor-α, *MMP* matrix metalloproteinases, *LIF* leukemia inhibitory factor, *SCF* stem cell factor, *M-CSF* macrophage colony-stimulating factor, *FGF-2* fibroblast growth factor-2, *MCP-1* monocyte chemoattractant protein-1, *PIGF* placenta growth factor, *PGE-2* prostaglandin E2, *TGF-β* transforming growth factor-β, *IDO* tryptophan catabolizing enzyme indoleamine 2,3-dioxygen, *iNOS* inducible nitric oxide synthase, *HSC* hepatic stellate cells, *ECM* extra cellular matrix

curing different types of conditions, from cardiovascular diseases to those affecting the kidneys, liver, and pancreas. Even more intriguing is the fact that, while some trials implicate the importance of MSC differentiation (i.e., for skeletal diseases), most of the trials seem to prevalently rely on the paracrine effects of MSCs rather than on their differentiation abilities, therefore highlighting this new possible repertoire for therapy (for an update, see [145]).

Little is known regarding the in vivo survival of MSCs after transplantation or their possible long-term adverse effects, such as ectopic tissue formation, malignant transformation, and immunogenicity. In this regard, Breitbach and co-workers have observed calcifications in the infarcted hearts of mice that had received local MSC treatment [146]. Meanwhile, although no in vivo transformation or tumor formation has been observed in MSC-treated patients, considering that most in vivo applications using MSCs are performed using in vitro cultured and expanded cells, we cannot exclude the possibility that such in vitro manipulation may negatively alter the characteristics of these cells and induce a malignant transformation in vivo [147–149]. Finally, although MSCs are considered to have absent or low immunogenicity, recent evidence indicate that, under appropriate conditions, these cells can function as antigen-presenting cells and activate immune responses, thereby eliciting their rejection [150–152]. Therefore, further controlled studies are required to address these concerns regarding the safety of MSC for development of cell therapy approaches.

Conclusions

From this *excursus* of the current literature in the field, it is evident that the range of potential applications of MSCs in cell-based therapeutic approaches has evolved and broadened to include not only their ability to replace cells through differentiation but also on their ability to secrete biologically active molecules that exert beneficial effects on other cells and on the microenvironment which they occupy [20, 153].

Although many of the observations from preclinical models that support the beneficial effects of MSC-based approaches represent something of a "jigsaw puzzle," with many pieces still to be put together, and despite the many gaps remaining in our understanding of the mechanisms involved and possible long-term consequences of MSC transplantation, scientists continue to pursue clinical experiences and to test innovative approaches using these cells.

Meanwhile, although the original optimism for application of MSCs for tissue regeneration (regenerative medicine) has decreased in recent years, we are now beginning to appreciate a new facet regarding the potential of these cells in the clinical arena, with the concept of reparative medicine versus regenerative medicine. While the application of MSCs along either of these two lines entails the employment of differing logics and the design of different therapeutic protocols, future studies will no doubt show the importance, to differing degrees, of both of these aspects in the development of MSC-based cell therapies for treating a wide range of human conditions.

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The Role of Microenvironment Stromal Cells in Regenerative Medicine

2

Ian McNiece

Introduction

The homeostasis of cellular content is an ongoing process in most tissues, representing a balance between cell death and new cell production. One of the best studied systems in the body is the hematopoietic system which requires ongoing blood cell production due to blood cell turnover. The bone marrow (BM) is the principal site for blood cell formation in humans. In normal adults, the body produces about 2.5 billion red blood cells (RBC), 2.5 billion platelets, and ten billion granulocytes per kilogram of body weight per day [1]. All of these cells originate from hematopoietic stem cells (HSCs) which reside in the BM in close proximity to the microenvironment which nurtures the HSCs by providing growth factors and cytokines which control proliferation and differentiation. Deficiencies in the microenvironment at a cellular or molecular level result in abnormal cell production resulting in anemia, leukemia, or embryonic lethality [2]. Recent reports have demonstrated the presence of stromal cells in other tissues and organs including the heart, liver, and adipose tissue [3]. Mesenchymal stem cells (MSCs) are one of the cell types which make up the BM microenvironment, and similar cells have been identified in the other tissues which express similar surface markers, including CD105, CD90, and CD73 but negative for hematopoietic markers such as CD45 and CD34 [4]. In addition, the cells have a similar morphology and appearance in culture. However, gene expression studies demonstrate differences at the molecular level and suggest distinct gene profiles that may provide biologic insights into differences in tissue properties and biology.

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The BM from a wide range of mammalian species contains precursor cells that generate adherent colonies of stromal cells in vitro. The BM stroma represents the non-hematopoietic connective tissue elements that provide a system of structural support for developing hematopoietic cells. The complex cellular composition of marrow stromal tissue comprises a heterogenous population of cells including reticular cells, adipocytes, osteogenic cells near bone surfaces, vascular endothelial cells, smooth muscle cells in vessel walls, and macrophages [5, 6]. The concept that adult hematopoiesis occurs in a stromal microenvironment within the BM was first proposed by Dexter and colleagues, leading to the establishment of the long-term BM culture (LTMC) [6]. These studies demonstrated that an adherent stromal-like culture could support maintenance of hematopoietic stem cells (HSCs) [6]. Mesenchymal stem cells (MSCs) represent the major stromal cell population in the BM.

Mesenchymal Stem Cells (MSCs)

MSCs were recognized by Friedenstein who isolated cells from guinea pig bone marrow which were adherent in culture and which differentiated into bone [7]. Surface antigens have been reported for identification and phenotyping of human MSCs [8–10]. Although MSCs are rare, representing approximately 0.01 % of the bone marrow mononuclear cell fraction, they have attractive features for therapy, including the ability to expand many log-fold in vitro and unique immune characteristics allowing their use as an allogeneic graft. They are typically isolated based upon adherence to standard tissue culture flasks. Low-density BM mononuclear cells (MNCs) are placed into culture in basal media plus FCS (typically 10–20 %) and after 2–3 days adherent cells can be visualized on the surface of the flask. The nonadherent cells are removed at this time and fresh media added until a confluent adherent layer forms. The MSCs are harvested by treatment with trypsin and further passaged expanding the number of MSCs. A number of different cell populations

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have been isolated using different culture conditions; however, the morphology of these cells is very similar. Phenotypical characterization of MSC has been performed by many groups, and a standard criterion has been proposed by the International Society of Cellular Therapy (ISCT) [4]. The minimal criteria proposed to define human MSC by the Mesenchymal and Tissue Stem Cell Committee of the ISCT consists of the following: (1) the MSC must be plastic adherent when maintained in standard culture conditions; (2) MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19, and HLA-DR surface molecules; and (3) MSC must differentiate into osteoblasts, adipocytes, and chondrocytes in vitro [4].

A standard in vitro assay for MSC is the colony-forming unit fibroblast (CFU-F) assay [7]. BM MNCs are plated at low density, and colonies of fibroblasts develop attached on the surface of the culture dish. Based upon the results of this assay, the frequency of MSC precursor cells is one in 10^4 to 10^5 BM MNC. The frequency is highly variable between individuals, and the number of MSC has been shown to be decreased in older people. Other studies have demonstrated that MSC precursors can be isolated based upon surface antigen expression. Antibodies to CD271 and Stro-1 have been used to enrich MSC precursors. CD271, also known as low-affinity nerve growth factor receptor (LNGFR) or p75NTR, belongs to the low-affinity neurotrophin receptor and the tumor necrosis factor receptor superfamily. Selection of CD271+ cells from human BM enriches CFU-F, and MSCs are preferentially selected in the CD271+ fraction compared to the CD271- fraction [8, 9]. Similarly, isolation of Stro-1+ cells from BM MNC results in enrichment of CFU-F in the Stro-1+ fraction compared to the Stro-1- fraction [10].

Immunologic Properties of MSCs

MSCs are ideal candidates for allogeneic transplantation because they show minimal MHC class II and ICAM expression and lack B-7 co-stimulatory molecules necessary for T-cell-mediated immune responses [11–13]. Indeed MSCs do not stimulate a proliferative response from alloreactive T cells even when the MSCs have differentiated into other lineages or are exposed to proinflammatory cytokines [13]. As previously reviewed [14], MSCs have significant immunomodulatory effects, inhibiting T-cell proliferation [15], prolonging skin allograft survival [16], and decreasing graft-versus-host disease (GVHD) [17]. Recently human MSCs were shown to alter the cytokine secretion profile of dendritic cells, T cells, and natural killer cells in vitro, inhibiting secretion of proinflammatory cytokines (e.g., TNF-a, IFN-γ) and increasing expression of suppressive cytokines (e.g., IL-10), possibly via a prostaglandin E2-mediated pathway [18]. In vivo studies of the fate of MSCs have shown that, when transplanted into fetal sheep, human MSCs engraft, undergo site-specific differentiation into various cell types, including myocytes and cardiomyocytes, and persist in multiple tissues for as long as 13 months after transplantation in non-immunosuppressed immunocompetent hosts [19]. Further, in vivo studies using rodents, dogs, goats, and baboons demonstrate that allogeneic MSCs can be engrafted into these species without stimulating systemic alloantibody production or eliciting a proliferative response from recipient lymphocytes [20–23]. These properties present MSC as a promising source of allogeneic cells for tissue repair.

The Stem Cell Niche

The control of proliferation and differentiation of a number of types of stem cells (SCs) occurs in the microenvironmental niche or the stem cell niche. Hematopoietic stem cells (HSCs) have been studied in detail and shown to reside in the bone marrow in association with stromal cells which make up the hematopoietic microenvironment [24]. The stroma consists of several cell populations including mesenchymal stem cells (MSCs), fibroblasts, and adventicular reticulocytes [1]. HSCs exist in a quiescent state in close relationship with the stromal cells in the bone marrow. These stromal cells produce a number of cytokines and growth factors that are either secreted or expressed as membrane bound proteins, and these cytokines and growth factors control the differentiation and proliferation of the HSC. In vitro, MSCs have been shown to support the proliferation and differentiation of HSC, generating committed hematopoietic progenitor cells over a 6-week period [6]. If the microenvironment is compromised, such as in patients who receive multiple rounds of high-dose chemotherapy regimens, normal homeostasis is disrupted, and deficiencies in blood cells occur.

Stromal Cells in Cardiac Tissue

The extracellular matrix (ECM) of cardiac tissue provides elasticity and mechanical strength. The cardiac ECM is composed of a number of cells including cardiac fibroblasts, mesenchymal cells, fibronectin, and other matrix proteins [25–27]. We have isolated several stromal cell populations from human fetal heart which are positive for CD105, CD90, and CD73 but negative for CD34 and CD45, which is consistent with the phenotype of BM-derived MSC [4, McNiece I, 2012]. Given the homeostatic role of MSC in regulation of HSC, it is highly likely that cardiac stromal cells play a regulatory role in the control of proliferation and differentiation of cardiac stem and progenitor cells (CSC and CPC). This role could be performed through the secretion of a range of growth factors and cytokines.

MI results in ischemic damage which results in cell death of not only cardiomyocytes but also fibroblasts and most likely stromal cells. Even with migration of viable CSCs and

CPCs to the ischemic tissue, the lack of stromal elements would result in the failure of the CSCs and CPCs to proliferate and differentiate, hence failure of remodeling. Along with the recent identification of cardiac stem cells in heart tissue, this offers insights into the biology of ischemic heart damage. Patients with an MI have ischemic tissue which fails to regenerate, and we propose that this is in part due to destruction of cardiac stromal cells.

MSCs derived from BM cells have been evaluated for cardiac regenerative therapy [28] and offer advantages over other sources of stem cells because of their availability, immunologic properties, and record of safety and efficacy. Studies of MSC engraftment in rodent and swine models of myocardial infarction demonstrate (1) functional benefit in post-myocardial infarction (MI) recovery with administration, (2) evidence of neoangiogenesis at the site of the infarct, (3) decrease in collagen deposition in the region of the scar, and (4) some evidence of cells expressing contractile and sarcomeric proteins but lacking true sarcomeric functional organization. Administration of autologous or allogeneic human MSCs to cardiovascular patients has been performed in several clinical studies to date, all in the post-myocardial infarction (MI) setting. The MSCs have been administered via the intracoronary route (IC), via peripheral intravenous (IV) injection or direct injection into the cardiac tissue with surgery.

The mechanism of action of MSCs in cardiac repair has been studied, and in part infusion of MSCs facilitates migration of endogenous c-kit+ cardiac stem cells to the infarct region resulting new cardiomyocyte production [29]. Based upon these data, we propose that optimal repair of ischemic tissue requires regeneration of both stromal elements and cardiomyocytes. Delivery of MSC to the ischemic tissue can regenerate the stroma, and delivery of cardiac stem/progenitor cells can regenerate cardiomyocytes. We further propose that the combination cellular therapy is necessary for optimal repair as delivery of cardiac stem/progenitor cells will result in minimal repair due to the lack of a niche and the absence of appropriate growth factors and cytokines for these cells to proliferate and differentiate.

Characterization of Cardiac Stromal Cells (CStrCs)

We have isolated 20 different CStrC lines from human heart tissue, and in all cases, flow cytometry analysis demonstrated an equivalent phenotype to BM MSC with positive staining for CD105, CD73, and CD90 and negative for CD45 and CD34. BM MSCs were negative for CXCR4; however, 5–10 % of CStrCs were positive for CXCR4 expression. The CStrCs formed CFU-F colonies when plated in vitro, with a median of 26 (range 17–32) CFU-F per 100 cells plated. In contrast, BM MSCs contained fewer CFU-F ($p<0.01$), forming only three (range 2–5) CFU-F per 100 cells plated. In addition, the rate of proliferation of CStrCs was higher than BM MSCs reaching confluency within 3–4 days compared to 7–10 days for BM MSCs when seeded at identical cell doses. These data suggested that although the CStrCs were similar to BM MSCs, they may be different at a molecular level. Therefore, we performed a global gene array analysis and a microRNA array (miR). The MSC and stromal cells were culture expanded and then RNA prepared for microarray analysis. This analysis demonstrated distinct gene patterns between these two sources of stromal cells. In particular, distinct cytokine and cytokine receptor patterns (Table 2.1a) but also adhesion molecules (Table 2.1b). These data are suggestive of stromal cells in different tissues secreting different cytokines, and the expression of different cytokine receptors indicates that it can be influenced by tissue-specific signals. The increased expression of myosin genes and laminin alpha 5 would be consistent with cardiac expression compared to bone marrow-derived cells.

Table 2.1a Cytokines and cytokine receptors expressed at twofold or higher levels in cardiac stromal cells compared to bone marrow MSC

3588	IL10RB – interleukin 10 receptor, beta
4982	TNFRSF11B – tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
7048	TGFBR2 – transforming growth factor, beta receptor II (70/80 kDa)
51330	TNFRSF12A – tumor necrosis factor receptor superfamily, member 12A
3460	IFNGR2 – interferon gamma receptor 2 (interferon gamma transducer 1)
355	FAS – Fas (TNF receptor superfamily, member 6)
5156	PDGFRA – platelet-derived growth factor receptor, alpha polypeptide
58191	CXCL16 – chemokine (C-X-C motif) ligand 16
2690	GHR – growth hormone receptor
7424	VEGFC – vascular endothelial growth factor C
1436	CSF1R – colony-stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
8764	TNFRSF14 – tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)
10344	CCL26 – chemokine (C-C motif) ligand 26
4233	MET – met proto-oncogene (hepatocyte growth factor receptor)
3590	IL11RA – interleukin 11 receptor, alpha
3953	LEPR – leptin receptor

Table 2.1b Adhesion molecules expressed at twofold or higher levels in cardiac stromal cells compared to bone marrow MSC

5293	PIK3CD – phosphoinositide-3-kinase, catalytic, delta polypeptide
4638	MYLK – myosin, light polypeptide kinase
894	CCND2 – cyclin D2
3911	LAMA5 – laminin, alpha 5
29895	MYLPF – fast skeletal myosin light chain 2
3672	ITGA1 – integrin, alpha 1
3371	TNC – tenascin C (hexabrachion)
9379	NRXN2 – neurexin 2
3689	ITGB2 – integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
7412	VCAM1 – vascular cell adhesion molecule 1
4267	CD99 – CD99 molecule
3134	HLA-F – major histocompatibility complex, class I, F
3133	HLA-E – major histocompatibility complex, class I, E
3385	ICAM3 – intercellular adhesion molecule 3
3384	ICAM2 – intercellular adhesion molecule 2
3108	HLA-DMA – major histocompatibility complex, class II, DM alpha
23114	NFASC – neurofascin homolog (chicken)

Table 2.2 MicroRNAs expressed at higher or lower levels in cardiac stromal cells compared to bone marrow MSC

miR	Fold up	miR	Fold down
miR-1	63	miR-10b	9
miR-10a	11	miR-335	13
miR-15a	3	miR-451	13
miR-15b	6	miR-479	8
miR-16-1	16	miR-628-3p	9
miR-18a	24	miR-768-3p	2
miR-19b-1	15		
miR-20a	16		
miR-26a-2	7		
miR-33a	26		
miR-92a-1	137		
miR-133a	57		
miR-206	1,181		
miR-374a	4		
miR-411	10		
miR-424	2		
miR-450a	2		
miR-450b-5p	37		
miR-454	3		
miR-503	35		
miR-516a-3p	2		
miR-542-3p	3		
miR-551b	5		
miR-589	5		
miR-770-5p	2		
miR-935	213		

Genes expressed at twofold higher (upregulated) or lower (downregulated) levels in CStrCs compared to BM MSC are presented

There were 26 miRs upregulated and six downregulated by more than twofold in CStrCs compared to BM MSC (Table 2.2). Three (miR-1, miR-133a, and miR-206) of the upregulated miRs have functions in myogenesis and cardiomyocyte development [30], and two (miR-20a and miR-26a) have function in stem cell differentiation [31, 32]. Of particular note was miR-206, which was upregulated by more than 1,000-fold in CStrCs. miR-206 has been reported as a muscle-specific miR that promotes muscle differentiation and regulates connexin 43 expression during skeletal muscle

development [33, 34]. In addition, miR-1 and miR-206 have been reported to be upregulated in the heart following myocardial infarction compared to normal heart [35].

A previous report by Rossini and colleagues [36] compared human BM stromal cells to CStrCs and also found an identical phenotype between the two cell populations. Interestingly, the differentiation potential of the two cell populations was different with CStrCs failing to express alkaline phosphatase when cultured in osteogenic medium, but the CStrCs acquired endothelial markers including CD144, VEGFR2, and CD31 and formed capillary-like structures at higher levels than BM-derived stromal cells. The studies by Rossini and colleagues also reported differences in microRNA expression with higher expression of miR-146a, mir-146b, and mir-330 in CStrCs compared to BM stromal cells. The increased expression of miR-146a, 146b, and 330 in CStrCs is not consistent with any previous reports of a role of these microRNAs in cardiac tissue. In contrast, our data defines several microRNAs with previously reported roles in cardiac tissue.

In addition to the role of miR-206 in skeletal and cardiac muscle development, this miR has been implicated in repression of tumor cells. Specifically miR-206 has been shown to repress estrogen receptor-alpha signaling in breast cancer cells [37] and suppresses breast cancer metastasis [38]. Also, miR-206 blocks human rhabdomyosarcoma growth by promoting myogenic differentiation [39]. As tumor formation is rare in cardiac tissue, it is possible that miR-206 may play a role in blocking circulating tumor cells from seeding and proliferating in cardiac tissue. This observation may have a significant impact on deciding what source of stromal cells to use in regenerative medicine approaches. Many current ongoing clinical studies utilize BM MSCs, which are supportive of tumor cell development with leukemias and lymphomas developing in the BM and have been shown to migrate to sites of tumor formation and facilitate vascular growth in tumor mass in vivo [40]. It is possible that BM MSCs injected into cardiac tissue may facilitate tumor formation in the heart. In contrast, CStrCs which express miR206 would not be permissive for tumor growth and maintain the integrity of the organ.

Summary

Although CStrCs have an identical phenotype to BM-derived stromal cells, they have distinct gene expression profiles. In particular, there are distinct expression profiles for cytokines and growth factors and microRNAs that could control differentiation of cardiac stem cells. Studies by Rossini and colleagues have demonstrated superior in vivo effects of CStrCs on repair and remodeling in rat MI model compared to BM stromal cells [41, 42]. These studies also demonstrated similar immunosuppressive properties of cardiac stromal cells to BM stromal cells and suggest that CStrCs could be a more effective cell population for cardiac repair than BM MSCs.

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Part II

Menstrual Blood Stem Cells

Identity of Human Endometrial Tissue: Potent Source of Stem Cells

3

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Introduction

The uterus is by far the largest female organ of the body, playing an integral role in the reproductive life of every woman. It plays a pivotal role in implantation and in absence of pregnancy, menstruation. The innermost layer of the uterus is known as tunica mucosa, popularly termed as endometrium, opposed to the outer perimetrium and median myometrium. The uterus is the only organ whose lining is almost entirely expelled and reconstructed periodically, both phenomena taking place at each ovarian cycle. With the purpose of facilitating the periodic elimination of the endometrium that undergoes regression, shrinkage, and necrosis at end of each cycle, the uterus also exhibits the unique peculiarity of physiological bleeding. The endometrial histophysiology is entirely controlled by the ovarian hormones along the cycle. Of all tissues of the human body, the endometrium is the one that, throughout the ovarian cycle, most accurately reflects the levels of estrogen and progesterone. Estradiol, produced by the ovaries on approximately day 4 or 5 of the cycle, induces growth and proliferation of the endometrium. The levels of estrogen are normally elevated during the proliferative phase of the menstrual cycle as it serves to promote proliferation of the luminal and glandular epithelial cells associated with the thickening of the endometrial lining as well as vascularization. The cessation of endometrial growth occurs before estradiol levels reach their peak and prior to the onset of progesterone production, thereby indicating that nonsteroidal factors limit the growth of endometrium. Progesterone is responsible for the secretory phase of the ovulatory cycle, and its action upon the endometrium serves two purposes. The first can be regarded as "medical." It greatly reduces the proliferative activity of the endometrial glands, thereby preventing the appearance of endometrial hyperplasic alterations. The second is essentially "reproductive," that is vital to create an ideal condition in the endometrium for the implantation and development of the egg [1–3].

Reproduction in the mammalian female thus requires a precisely timed and complex interaction between the hypothalamic-pituitary-ovarian (HPO) axis and the uterine endometrium. Abnormalities in HPO axis are being treated effectively with advancements in reproductive medicine. However, the abnormalities in endometrium function that serve as a major cause of poor implantation of the fetus in the uterine wall remain poorly understood. Abnormalities in endometrium also lead to female infertility and several pathological diseases, like endometrial cancer, endometriosis, endometrial hyperplasia, and endometritis, to name a few. Development of effective therapy for infertility or other diseases due to endometrial dysfunction requires enhanced understanding of the latest advance in uterine/endometrial cell biology, more precisely the discovery of endometrial stem cells.

This chapter provides a brief overview on the current understanding of the evidence supporting the existence of the uterine adult stem cells in the endometrial tissue and the role these cells likely play in normal adult uterine physiology. We describe the isolation of endometrial stem cell, their propagation, their biomarker expression, and their differentiation potential. In addition, we review the possible roles in gynecological disorders associated with abnormal endometrial proliferation and the potential use of endometrial stem cells in therapeutics.

Endometrial Stem Cells: Research Background

Embryonic stem cells, the cells derived from the inner cell mass (ICM) of the developing embryo, are pluripotent and

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were considered an ideal source of regenerative therapeutics for treatment of a wide range of diseases. But ethical issues and immune rejection limit their clinical applications. Moreover, the tumorigenicity of embryonic stem cells is a concern [4–6]. Hence, there occurred a successful search for stem cells from adult tissues and organs. Bone marrow and adipose tissue are promising sources of adult stem cells and are now being used in a wide range of therapies [7–13]. This revolutionary change has increased the demand of identification of stem cells from other tissues and body fluids such as tendon [14], periodontal ligament [15], synovial membrane [16], lung [17], liver [18], synovial fluid [19], amniotic fluid [20–22], as well as endometrial tissue [23] and menstrual blood [24, 25]. Despite the advances in recent years in the isolation and applicability of stem cells from many sources, there exist only limited studies that consider the availability and applicability of stem cells in female and male reproductive organs. Studies of adult stem cell biology in the uterus lag far behind other areas of stem cell research despite the fact that the uterus undergoes perhaps the most extensive proliferative changes and remodeling in adult mammals compared with other organs.

Human endometrium lines the uterine cavity as far the isthmus of the uterus, where it becomes continuous with the lining of the cervical canal. The endometrium begins to reach full development at puberty and thereafter exhibits dramatic changes during each menstrual cycle. It undergoes further changes before, during, and after pregnancy, during the menopause, and in old age. The endometrium is a simple columnar epithelium. It is divided into two zones, the inner functionalis which is adjacent to the uterine cavity and a deeper basalis layer which overlies the myometrium. The functionalis layer is shed each month with menstruation and is then regenerated from the basalis layer which is not shed. The functionalis, comprising the upper two-thirds of the endometrium, is divided into stratum compactum and stratum spongiosum. The stratum compactum is a superficial thin layer nearest to the uterine cavity and contains the lining cells, necks of the uterine gland, and relatively dense stroma. The stratum spongiosum is the deeper part of functionalis composed of main portions of the uterine glands and accompanying blood vessels; the stromal cells are more loosely arranged and larger than in the stratum compactum. The lower basalis contains the basal region of the uterine glands, dense stroma (that remains relatively unaltered during the menstrual cycle), large blood vessel remains and lymphoid aggregates. It serves as the germinal compartment for generating new functionalis each month [26–28]. It has been postulated that the niche of these adult stem or progenitor cells of the endometrium is the lower basalis. These stem or progenitor cells were also identified to be in the trophic endometrium of postmenopausal women [29, 30].

Accumulating evidence from the literature on the existence of epithelial and stromal/stem cells in endometrial tissue [28–31] has substantiated that it possesses a remarkable capacity for regeneration. However, there is only limited information pertaining to these stem cells derived from endometrial tissue as obtaining these stem cells directly from the endometrial tissue (the inner lining of the uterus) requires an endometrial biopsy or uterine curettage. Endometrial biopsies are routinely performed for diagnosis of abnormal uterine bleeding and can be done easily in an office setting with a small curette or the self-contained small vacuum cannula (Pipelle™) which minimizes the patient's discomfort. Each menstrual cycle is associated with the vascular proliferation, glandular secretion, and the endometrial growth. Absence of progesterone, the demise of corpus luteum, and the subsequent fall in circulating progesterone lead to vasoconstriction, necrosis of the endometrium, and menstruation. Menstrual blood includes the apical portion of the endometrial stroma. Hence, menstrual blood has become the most convenient source in the search for endometrial stem cells because collecting menstrual blood is easy and noninvasive and endometrial stem/progenitor cells are shed in menstrual blood. For these reasons, reliable studies on menstrual blood-derived stem cells are in process [32–35]. Furthermore, menstrual blood-derived stem cells demonstrate great promise for use in tissue repair and treatment of diseases, due to the plasticity and longevity of the cells. This has been identified through the in vitro and in vivo studies of characterization, proliferation, and differentiation [23, 25, 34].

However, putative adult stem or progenitor cells that are responsible for the cyclical regeneration of the endometrium functionalis, every month, are thought to reside in the basalis region of the endometrium [26–30]. Hence, the study of these stem cells from the basalis layer of the endometrial tissue is of utmost importance and is still in its infancy. Based on the dynamic tissue remodeling in all compartments of the uterus, during the menstrual cycle and pregnancy, it has been suggested that adult stem cells must play a role in uterine tissue maintenance and function. Hence, a thorough characterization of the uterine/endometrial stem cells derived from the endometrial tissue biopsy of the inner lining of the uterus or from the intact uterus surgically removed in the treatment is equally important as that of studies on menstrual blood stem cells. Once a mechanical or functional characteristic platform has been constructed, it then becomes easier to understand the complex mechanisms underlying the morphogenesis and physiological generation of the female reproductive tract, to improve the understanding of the pathophysiology of the gynecologic diseases such as endometrial cancer, fibroids, endometriosis, and pregnancy loss as well as determine the possible roles of endometrial stem/progenitor cells of the female reproductive tract to these gynecologic diseases, thereby considering them as a possible therapeutic target for treatment of wide horizon of diseases in regenerative medicine.

Evidence for Endometrium Stem Cells

Adult stem cells in the endometrium are difficult to identify because they constitute very small populations of cells and because of the lack of precisely characterized cell surface markers specific for adult stem cells of the endometrium. Studies which provide indirect evidence for the existence of endometrial stem cells do so by characterizing cell populations in the endometrium which exhibit the functional properties of stem cells. These properties include clonogenicity, proliferative potential, and capacity for differentiation into one or more lineages [29]. Clonogenicity, defined as the ability of a single cell to produce a colony when seeded at very low densities, was demonstrated in human endometrium for the first time in 2004. The first published evidence on stem cells of human endometrium identifies two types of adult stem cells, clonogenic epithelial and stromal cells suggesting the presence of two types of adult stem/progenitor cells [36, 37]. Using a purified single cell suspension dispersed from hysterectomy specimens, Chan et al. identified a small population of stromal cells (1.25 %) and epithelial (0.22 %) cells in human endometrium that possessed clonogenic activity [37]. This rare population of epithelial and stromal colony-forming unit cells (CFUs) were found in normal cycling and inactive perimenopausal endometrium and in endometrium of women on oral contraceptives, suggesting that CFUs may be responsible for regenerating cyclic and atrophic endometrium [38, 39]. The findings, however, of clonogenic cells in inactive endometrium further supports the existence of an endometrium stem cell niche in the basalis, as this endometrium is predominantly basalis and lacks functionalis. Only large CFUs exhibit stem cell properties of self-renewal, differentiation, and highly proliferative potential, while small CFUs are transit-amplifying cells [24].

Another approach used by multiple investigators was to identify and characterize stem cells with the side population phenotype (SP). Side population cells are characterized by their ability to exclude the DNA binding dye Hoechst 33343 by expressing ATP-binding cassette transporter proteins. They exhibit the properties of adult stem cells including mature glandular epithelial, stromal, and endothelial cells in vitro. The SP phenotype cells have also been found to proliferate and differentiate into requisite cell types in vivo in the immunodeficient mice [40]. This method has been previously used to identify putative stem cell population in multiple tissues, including the bone marrow, liver, mammary gland, skin, and kidney, inclusive of endometrium. Several groups have identified a number of SP cells as candidate endometrial stem/progenitor cells. SP cells identified from human endometrium display long-term proliferative properties as well as differentiation into mature endometrial glandular, epithelial, stromal, and endothelial cells in vitro [41–43]. Additional studies have reported the ability of endometrial SP to differentiate in vitro into adipocytes and chondrocytes, thus supporting a mesenchymal origin [44, 45]. These studies support the hypothesis that SP isolated from human endometrium are indeed adult stem cells. Other properties evaluated in characterization of an endometrial stem cell population include the capacity of multilineage differentiation. The differentiation potential of candidate stem cells is evaluated after culturing the cells in differentiation induction media. The endometrial stem cells were able to differentiate into muscle cells, adipocytes, osteoblast, and chondrocytes [44–48].

Schwab and Gargett demonstrated the existence of endometrial stem cell identification through the characterization of perivascular markers CD 146 and PDGF-Rβ. They demonstrated that these perivascular markers enabled isolation of stromal cells from human endometrium which exhibit phenotypic and functional properties of MSC. The investigators then used immunohistochemistry to localize these cells to perivascular areas of the basalis and functionalis [48]. They hypothesized that these endometrial MSC-like cells may contribute to the cyclic regeneration of the endometrium and further postulated that they may play a role in pathogenesis of diseases such as endometriosis and adenomyosis. Despite the scanty citations on the expression profile of biomarkers of endometrium stem cells [49], the identification of other prospective markers to identify endometrial stem cells is underway.

Isolation and Propagation of Stem Cells Derived from Endometrial Tissue

Endometrium was collected from reproductively active women undergoing hysterectomy or endometrial biopsy for nonmalignant uterine tumors, fibroids, adenomyosis, and uterine prolapse. Endometrial tissue was collected from the different phases of endometrial cycle including menstrual, secretory, and proliferative phase. Sampling procedures varies with the aim of research, but samples were usually collected from women not under any kind of hormonal therapy [24, 49–51]. The endometrial biopsy samples containing the endometrial epithelial and stroma cells are collected in HEPES-buffered Dulbecco modified Eagle medium/Hams F-12 supplemented with antibiotic-antimycotic solution (final concentrations: 100 mg/ml penicillin G sodium, 100 mg/ml streptomycin sulfate, 0.25 mg/ml amphotericin B) and newborn calf serum [37] or Hanks balanced salt solution containing the antibiotics like streptomycin and penicillin [50].

Isolation of endometrium stem cells involves processing of the finely chopped tissue samples in phosphate-buffered saline devoid of calcium and magnesium ions. The mechanically minced tissue is further digested with collagenase type

III and deoxyribonuclease type I to prevent increase in viscosity of the cell suspension due to nucleic acid [37] or DMEM containing type Ia collagenase [50]. The stromal cell suspension is obtained and purified either by negative selection using magnetic Dynabeads coated with specific antibodies to remove epithelial cells (BerEP4) and leukocytes (CD45) [37] or by repeated centrifugation to obtain the epithelial cells and the stromal cells [50]. The stromal isolates are cultured and propagated in the complete culture medium.

Although the freshly isolated samples possesses the heterogenous mixture of both epithelial and stromal cell populations, a homogenous fibroblastic stromal population becomes prominent with a spindle-like cell morphology with centrally located nuclei as a monolayer upon culture [50]. The adult stem cell properties were also assessed by separating into $EpCAM^+$ epithelial cells and $EpCAM^-$ stromal cells. The self-renewal property of the adult stem cells was also monitored using a serial cloning strategy. Large and small clones were obtained wherein the large clones showed a better self-renewal capacity. Large clones of epithelial and stromal cells were capable of undergoing three rounds of serial cloning [24]. The percentages of colony-forming mesenchymal cells from endometrium were found to be higher when compared to bone marrow or dental pulp. Human endometrial stromal cells were found to have a colony-forming capacity of 1.2 % in comparison to the 0.1–0.01 % for dental pulp and bone marrow [51].

Biomarker Expression

A great breakthrough has been achieved by the identification and isolation of the stem cells from endometrium. However, the search to identify the MSC population in human endometrium is still at its infancy [52]. Furthermore, there exist only scanty citations on identification of biomarker expression of the uterine/endometrial stem cells that can isolate/characterize specific cell population. A major advantage of being able to identify the cell surface markers of epithelial and stromal population of the endometrium is that their features can be characterized in noncultured cells and their utility in cell-based therapies for regenerative medicine evaluated in preclinical disease models. Furthermore, a detailed study of endometrial stem cell markers is necessary as pathology of several endometrial disorders is associated with these endometrial progenitor/stem cells.

The presence of mesenchymal stem cells can be confirmed by surface antigenic profiling. A positive expression of markers like CD105, CD73, and CD90 and the absence of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules define mesenchymal stem cells as proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) [53]. These criteria help researchers to identify mesenchymal stem cells and progenitors derived from endometrium as well as their expression prevalence in endometrium associated diseases. Dimitrov and his co-workers [50] analyzed the cell surface markers for the cultured endometrial stem cells derived from both functionalis and basalis layer of the endometrium for phenotypic expression. Hematopoietic stem cell markers including CD 45, CD 14, CD 19, CD56/16, CD 34, and CD3 showed a negative expression. Whereas markers like CD 29, CD 73, and CD 90 were stained positive, strongly suggesting the mesenchymal nature of the cells. In addition, immunofluorescence staining of the stromal cells showed positivity for vimentin, prometalloprotinease-3 (proMMP 3), and endoglin (CD 105) showing their respective cellular localization.

Schwab and his co-workers identified the co-expression of perivascular cell markers like melanoma cell adhesion molecule (MCAM)/CD 146 and platelet-derived growth factor receptor β (PDGF-Rβ) using a fluorescence-activated cell sorter (FACS). The FACS-sorted $CD146^+PDGF\text{-}R\beta^+$endometrial stromal cell population was found to have a positive phenotypic expression of mesenchyme-specific markers CD29, CD44, CD73, CD90, and CD105 and a negative expression of hematopoietic markers CD 34 and CD 45 as well as endothelial marker CD 31. This study also reveals that CD 146 and PDGF-R β cells were co-localized in both the functional and basal layer. Moreover, the conclusions imply that a few $CD146^+PDGF\text{-}R\beta^+$ cells may shed during menstrual cycle [48].

Schwab and his co-workers studied a sorting strategy to isolate human endometrial stromal stem cells. In this screening investigation, fresh endometrial stromal cell suspension was studied for the expression of the surface proteins, STRO-1, CD 133, CD 146, and CD 90, using immunohistochemistry, FACS sorting, and CFU assays [49]. Thy-1 (CD 90) was used in conjugation with CD 45 (negative marker) which led to the enrichment for CFU in CD90 stromal cell population. Along with CD 90, CD146 was also found to enrich human endometrial CFU, suggesting the perivascular location of these cells. This result was in accordance with the co-expression of CD 146 with platelet-derived growth factor receptor β (PDGF-Rβ) [48] and thus favors the isolation of mesenchymal stem cells from human endometrium. Even though the perivascular endometrial cells expressed STRO-1, these cells failed to enrich CFU thereby making them not useful for isolating human endometrial stem cells. Likewise, HSC marker CD 133 turned out to be a negative marker for stromal endometrium CFU. This study clearly suggests the utility of CD 146 and CD 90 as potential markers for the sorting strategy of endometrial CFU [49].

Octamer 4 (OCT-4) a highly expressed transcription factor in embryonic stem cells and in embryos at various stages of development was also studied by researchers on endometrium. OCT-4 expression was evident only in the endometrial

stromal cells and not in the myometrium. The study indicated that OCT-4 expression does vary with age, with phase of the menstrual cycle, or with the gynecologic disorders of the individual. The expression of OCT-4 suggests the existence of endometrial stem cells [54], lending further support to the hypothesis of endometrial regeneration by local stem cells in endometrial tissue. A similar study reported the expression of OCT-4 in the follicular and luteal phases of endometrium by Bentz and his co-workers [55], thereby suggesting the presence of pluripotent cells in the endometrium. On the other hand, as OCT-4 expression has been previously associated with germ cell tumors and embryonic carcinoma [56], a thorough study on OCT-4 expression of the endometrial stem cells is needed and may help us to understand the pathology of endometrial cancers and other disorders associated with abnormal proliferation of endometrium.

Overall, there are only limited studies on phenotypic biomarker characterization since endometrial stem/progenitor cells were only identified in the year 2004. Albeit these limited studies, endometrial tissue-derived stem cell had gained importance because of its ease of availability and distinct biomarker expression identified so far. However, much more needs to be explored in identifying the other cell surface markers that are unique to epithelial and stromal cell populations of the endometrium.

Differentiation Potency

One of the unique characteristic features of stem cells is their ability to undergo multilineage differentiation. The mesenchymal and tissue stem cell committee of the International Society for Cellular Therapy (ISCT) has put forward certain minimal criteria to define and identify the MSC population [53]. One of the criteria is that the cells must be able to differentiate to mesodermal lineages of osteoblasts and adipocytes to demonstrate bone and fat phenotypes, respectively, under standard in vitro differentiating conditions [57]. Evidence on the existence of endometrial stem cells was derived from the phenotypic, functional, and proliferative studies. As per the criteria put forth by ISCT, it is imperative to understand whether the stem cells of endometrium have the ability to differentiate into multiple lineages as described for other stem cell types. Indeed several researchers demonstrated the ability of $CD146^{+}PDGFRb^{+}$ MSC-like cells [48] or clonogenic human endometrial stromal cells [24, 50] to differentiate into mesodermal origin such as adipocytes, osteocytes, smooth muscle cells, and chondrocytes. Masuda et al. demonstrated that the endometrial tissue-reconstituting cells also possess the ability to differentiate into endothelial cells [58]. It has been demonstrated that not only the endometrial stem cells but also the SP cells of the endometrium have the ability to differentiate into endothelial and smooth muscle cells [41–43]. Although accumulating evidence supports the ability of endometrial stem cells to differentiate into cells of mesodermal origin, the ability to transdifferentiate is yet to be defined. The possibility of these endometrial stem cells to differentiate into neuronal-like cells has been evaluated [59]. However, study of the transdifferentiation potential of these stem cells in near future might unearth the prospects of curative therapeutics.

Clinical Correlations and Regenerative Applications

Several gynecological conditions are associated with abnormal endometrial proliferation, and it is possible that putative endometrial stem/progenitor cells may play a role in the pathophysiology of diseases such as endometriosis, endometrial hyperplasia, endometrial cancer, and adenomyosis. Alterations in the number, function, regulation, and location of epithelial/stromal endometrial stem/progenitor cells may be responsible for any one of these endometrial diseases. Furthermore, study of the clinical correlations of endometrial stem cells with gynecological diseases may unravel several unresolved barriers and lead to the use of endometrial stem cells as an ideal alternative source of curative therapeutics.

Endometrial Stem/Progenitor Cells in Endometriosis

Endometriosis, defined as the growth of endometrium outside the uterine cavity, is a common gynecological disorder affecting 6–10 % of women [60]. It is a major clinical problem causing inflammation, pain, and infertility. Despite its common occurrence, the pathogenesis of endometriosis is poorly understood [61, 62]. The most widely accepted mechanism is Sampson's retrograde menstruation theory where viable endometrial fragments reflux through the Fallopian tubes into the pelvic cavity and attach to and invade the peritoneal mesothelium to establish ectopic growth of endometrial tissue. Several other theories have been suggested, including abnormal endometrium, genetic factors, altered peritoneal environment, reduced immune surveillance, and increased angiogenic capacity. It is proposed that endometriosis results when endometrial stem/progenitor cells are inappropriately shed during menstruation and reach the peritoneal cavity where they adhere and establish endometriotic implants [63]. Although no direct evidence exist on the role of endometrial stem cells in pathogenesis of endometriosis, numerous studies demonstrate that unfractionated human endometrial cells establish ectopic endometrial growth in the many experimental models [58, 64]. Despite the existence of

several other preliminary studies in vitro, the exact role of endometrial stem/progenitor cells in the development of endometriosis will require more elucidation.

Cancer Stem Cells in Endometrial Cancer

It is currently uncertain whether cancer stem cells have a role in endometrial cancers or in endometrial hyperplasia. Recent reports suggest a role of cancer stem cell on endometrial cancer. In a comprehensive study from both types and all grades of endometrial cancer, a small population of less than 1 % of clonogenic tumor cells were identified that could be serially cloned in vitro, indicating self-renewal capacity [65]. Freshly isolated endometrial cancer cells transplanted in limiting dilution into immunocompromised NOD/SCID mice induced tumors recapitulating parent tumor histoarchitecture and marker expression with as few as 1/10,000 in 50 % of transplants, indicating that a small population of tumor-initiating cells or cancer stem cells differentiated in vivo. SP cells have been identified in several low and high-grade endometrioid endometrial cancer samples and several endometrial cancer cell lines [66]. Interestingly, the Hecl SP cells produced tumors comprising epithelial tumor cells with the positive expression of human vimentin and alpha smooth muscle actin, indicating an occurrence of epithelial to mesenchymal transition cancer progression of the SP cells in the tumors in vivo. Furthermore, the role of cancer stem cells in endometrial cancers has also been proposed with the identification of other cancer stem cell markers responsible for endometrial cancer. The first of the surface markers used to identify the CSC in human endometrial cancer is the CD 133/1 epitope. In a larger study group of endometrial cancer, CD 133^+ cells have higher cloning efficiency and proliferated at a faster rate when compared to the CD 133− population [67]. It was hypothesized that the SP phenotype might also play a role in identification of CSC phenotype. Further studies of the CSC-specific marker in endometrium unravel its role in endometrium associated disorders.

Endometrial Stem Cells and Adenomyosis

Adenomyosis, a condition affecting 1 % of women, is characterized by the benign invasion of basal endometrial glands and stroma deep into the myometrium and is associated with smooth muscle hyperplasia. Little is known of the pathophysiology of adenomyosis. Adult stem cells are frequently activated in tissue injury, and it is possible that these have a role in establishing the ectopic lesions and their abnormal differentiation may be responsible for the smooth muscle hyperplasia [68]. Alterations in the orientation of endometrial stem/progenitor cells or their niche may be a major cause of the abnormal behavior in adenomyosis. The cells undergo differentiation toward the myometrium rather than toward the functionalis, producing pockets of endometrial tissue deep within the myometrium. It was recently shown that stromal cells cultured from adenomyotic tissue undergo multilineage mesodermal differentiation and express MSC surface phenotypic markers [57]. It is not known if these adenomyotic stromal cells are clonogenic, self-renew, or contain a population of CD 146^+PDGFRb^+ cells. This supports the idea that endometrial stem cells might be the major cause of adenomyosis. However, more research is required to establish this.

Unresolved Barriers and Concluding Remarks

Regardless of the strong evidence on the existence of adult stem cells in endometrium, the endometrial stem cell research is at its infancy. Major advances have been made to identify the populations of epithelial and stromal cells with stem/progenitor activity in human endometrium that is responsible for its remarkable regenerative capacity. Despite the current advancements, there still remain several unresolved barriers that hinder the potential applications of endometrial-derived stem cells in regenerative medicine. Whether these endometrial stem cells possess MSC activity in vivo and whether the cultures of menstrual blood/endometrial tissue-derived stem cells are equal has yet to be determined. Although certain markers of endometrial stem cells have been identified, there still remains a need for definitive markers of both endometrial stem/progenitor cells for more selective isolation, enrichment, and possible use in therapeutic approaches. Besides, complete phenotypic and functional characterization of uterine stem cells, inclusive of the menstrual blood and endometrial tissue-derived stem cells, will improve our understanding of the mechanism supporting physiological regeneration of the female reproductive tract. As endometrial stem cells become further better characterized, their role in gynecological disorders associated with abnormal endometrial proliferation can be assessed. Furthermore, these efforts have the potential to change the way these diseases may be treated in the future, particularly as therapeutic agents that target key stem cell functions becomes available.

Undoubtedly, it can be concluded that endometrial stem cells may become key players in regenerative medicine because of their noninvasive mode of collection, ease of isolation, its enhanced proliferative ability, and multiple differentiation potentials. Furthermore, the easy access to the endometrial stem cells through the menstrual cell mass and their potential for storage may allow greater applicability for cell therapeutics than other stem cells that are more difficult to obtain. Together, augmented studies of the characteristics

of putative uterine stem cells of endometrial tissue-derived and menstrual blood-derived stem cell population might determine how faulty adult stem cells of the uterus contribute to gynecological disorders as well as explore its applicability in cell-based therapies for treating a wide range of diseases.

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Endometrial Regenerative Cells and Exosomes Thereof for Treatment of Radiation Exposure

4

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In 2007, we discovered a novel subset of mesenchymal stem cells (MSCs) derived from the endometrium, termed "endometrial regenerative cells (ERC)." In comparison to other MSC types (e.g., bone marrow and adipose), ERC possess (a) more rapid proliferative rate, (b) higher levels of growth factor production (VEGF, GM-CSF, PDGF), and (c) higher angiogenic activity. We are currently running two clinical trials for these cells in patients with critical limb ischemia and heart failure.

The main cause of morbidity and mortality in patients suffering from acute radiation syndrome (ARS) is hematopoietic toxicity. Although ARS treatment is not part of routine medicine, our commercial interest lies in the ability to rapidly obtain FDA approval using the "Animal Efficacy Rule," which allows for developers of therapies used in disaster settings circumvention of Phase II and III trials if human clinical safety is established and efficacy is demonstrated in a relevant animal model.

Recent studies have demonstrated that BM-MSC are capable of preventing lethality subsequent to radiation exposure; however, these cells have performed poorly in late-phase trials. Given that ERC are substantially more economical to manufacture in large numbers and produce more hematopoietically relevant factors as compared to other MSC sources, we discuss the possibility of utilizing ERC as a cellular therapy for treatment of radiation exposure.

Exosomes are nanoparticles generated by a variety of cell types, implicated in cell-to-cell communication. MSC-BM exosomes have been shown to be a major mediator of MSC paracrine therapeutic effects. Our data demonstrate that ERC-generated exosomes stimulate BM mononuclear cell proliferation. We propose that administration of ERC-derived exosomes will increase postirradiation survival and hematopoietic recovery.

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Need for Effective Means of Treating Radiation Injuries

Protection against accidental or terrorist radiation exposure is attracting an increasing attention from military and civilian groups [1]. The possibility of nuclear war remains a reality: currently, there are approximately 30,000 nuclear warheads deployed around the world, 100 "suitcase bombs" unaccounted for, and attempts of terrorists to acquire a nuclear weapon, a "dirty bomb," or to attack a nuclear power plant or waste site. A Nuclear Regulatory Commission study stated that breaching a cask of spent fuel could release lethal radiation over an area many times larger than that affected by a 10 kt nuclear weapon [2]. Acute radiation syndrome (ARS), which is the main cause of morbidity and mortality associated with ionizing radiation exposure, is characterized by the triad of dysfunctions in the (a) neurovascular, (b) hematopoietic, and (c) gastrointestinal systems [3]. Intermediate-dose ARS, which is similar to that received by firefighters at the Fukushima Daiichi Nuclear Power Plant (3–7 Gy total body irradiation), is generally treated with hematopoietic growth factor support, whereas high-dose ARS (7–10 Gy) is treated experimentally with hematopoietic stem cell transplant [4]. Of the three systems that ARS targets, by far the most work has been performed in hematopoietic recovery with specific guidelines in place for administration of growth factors such as G-CSF and GM-CSF postexposure [3]. However, in addition to high cost, these factors are immunogenic and induce sites effects including bone pain. To date, with exception of potassium iodine, there is only one drug that has been FDA approved for postradiation exposure, amifostine, which acts as a DNA protectant and antioxidant [5]. Unfortunately, its administration is associated with a variety of adverse

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effects including hypotension in >60 % of patients, and its radioprotectant effects are limited in cases of myeloablative radiation [6]. Thus, alternative approaches are needed to support hematopoietic recovery in patients that receive intermediate or high doses of irradiation.

Mesenchymal Stem Cells (MSCs) Support Hematopoiesis

MSCs are known to contribute to the bone marrow hematopoietic microenvironment. Given that this microenvironment is disrupted by radiation damage [7, 8], studies were conducted to demonstrate that human MSC can accelerate hematopoietic reconstitution and/or recovery in animal models [9, 10]. Therapeutic activities of MSC are believed to occur by differentiating into cells of mesenchymal origin [11] and also through an indirect "chaperone" effect. This includes production of trophic/angiogenic factors, as well as anti-inflammatory/antioxidant properties [12, 13]. One interesting aspect of MSC is that production of growth factors such as IGF-1, VEGF, and HGF-1 seems to be upregulated by conditions associated with injury such as hypoxia [14] and inflammatory conditions [15, 16]. Supporting the possible use of MSC in treatment of radiation injuries are findings that MSC specifically home to areas of radiation exposure [17]. In preclinical studies, it has been demonstrated that human MSC administration enhances engraftment of human CD34 cells postradiation [10]. Accordingly, clinical implications of using MSC administration to enhance hematopoiesis were examined.

The original clinical use of expanded autologous MSC in 1995 demonstrated feasibility and safety of intravenous administration of these cells in 15 patients suffering from various hematological malignancies to prevent cytopenia [18]. In a subsequent study from the same group in 2000, the use of MSC to accelerate hematopoietic reconstitution was performed in a group of 28 breast cancer patients who received high-dose chemotherapy [19]. Donor MSCs were demonstrated to neutrophil and thrombocytic reconstitution in a post bone marrow transplant setting in a 46-patient trial [20]. In a similar study, Ball et al. reported on the use of purified donor-specific MSC (1–5 million/kg) being injected alongside with isolated CD34 from HLA-mismatched relatives in 14 pediatric leukemia patients. They showed that in contrast to traditional graft failure rates of 15 % in 47 historical controls, all patients given MSCs showed sustained hematopoietic engraftment without any adverse reaction [21]. The use of "third-party" MSC to enhance hematopoietic recovery was performed by Baron et al. in 20 patients who received non-myeloablative hematopoietic stem cell transplant, whose outcomes were compared to a historic control of 16 patients receiving a similar transplant protocol without MSC. Accelerated hematopoietic reconstitution and significant difference in 1-year survival (80 % vs 44 %) was noted [22]. These findings established a foundation for MSC-based therapies to be investigated clinically as augmenters of hematopoietic reconstitution and/or prevention of GVHD, with Phase II/III trials ongoing or having been completed [23].

MSC and Radiation Injury

In addition to preclinical and clinical data supporting the use of MSC in acceleration of hematopoietic reconstitution, several animal studies have formally studied ARS protection by MSC. Yang et al. demonstrated that a onetime infusion of either virally immortalized or primary mouse BM-MSC (one million cells per mouse i.v.) 24 h subsequent to 700 cGy X-radiation exposure led to a 53 % survival in mice receiving immortalized and 60 % survival for the group receiving primary MSC at 7 weeks post irradiation. All mice that were treated with vehicle control died [24]. Lange et al. obtained similar results in that administration of one million cloned or primary BM-MSC into lethally irradiated (9.5 Gy from cesium-137 source) 8 h after radiation resulted in 7-week survival of 66 % of treated animals, whereas 100 % of control animals died within 3 weeks. Although administered cells were localized primarily in the lung, microarray detection of gene expression in the bone marrow was noted, particularly, upregulation of genes associated with cell cycle and protection from oxidative stress, such as Cdkn1a and BRPK, as well as anti-inflammatory and detoxification genes Thbs2 and Gstm5. Survival was associated with reconstitution of endogenous hematopoiesis [25]. Similar results were reported by another group, in which it was demonstrated that BM-MSC infusion after sublethal irradiation (5.5 Gy) was associated with enhanced survival of BALB/c mice, as well as stimulation of bone marrow cell entry into cell cycle and reduction of apoptosis [26].

Non-hematopoietic Benefits of MSC Transplantation Following Irradiation

Although bone marrow failure is the major cause of morbidity and mortality, in "real-life" situations, ARS will be accompanied by GI failure, neurological consequences, pulmonary fibrosis, and possibility of multiorgan failure. Protection of the GI tract and 100 % 3-week survival subsequent to 10.4 Gy whole-body radiation exposure were demonstrated in mice treated with BM cells cultured in an MSC-differentiation media, whereas 100 % mortality occurred in controls [27]. MSCs have been demonstrated to be neuroprotective in models of stroke [28], intracerebral hemorrhage [29], as well as having the ability to stimulate endogenous neurogenesis [30]. Although to date studies on MSC prevention of radiation-induced neural damage have

not been performed, given that radiation inhibits endogenous neurogenesis [31], this is an appealing possibility. MSCs have been demonstrated to inhibit pulmonary fibrosis through anti-inflammatory mechanisms in several models [32]. Furthermore, multiorgan failure, whether induced by radiation or sepsis, presents with similar qualities. Inhibition of sepsis associated multiorgan failure has been demonstrated by BM-MSC and appears to function through an IL-10 and PGE-2-dependent pathway [33].

ERC as a Clinically Relevant MSC Population

Endometrial regenerative cells (ERC) were discovered by us in 2007 as a menstrual-blood-derived MSC population that possesses a higher proliferative rate (19–22 h), increased growth/angiogenic factor production, and longer passage ability as compared to BM-MSC [34]. These properties, as well as enhanced antifibrotic activities, were confirmed by two independent groups a year after [35, 36]. Currently, we are conducting a Phase I study in critical limb ischemia (NCT01558908) in the USA and a Phase II double-blind placebo-controlled cardiac study Ex-USA. To date, intrathecal [37], intramuscular [38], and intravenous administration [39] of the cells in pilot compassionate-use cases has revealed clinical safety of cell administration. This is relevant not only because of potential benefit based on enhanced antifibrotic and growth factor production properties of ERC but also due to low cost of isolation and mass production ($500 per clinical dose of 100 million cells).

The unique features of ERC made us examine their activity in an immunocompetent model of critical limb ischemia [40]. Subsequent to our publication, other groups have used these cells for treatment of stroke [41], Parkinson's disease [42], and diabetes [43, 44]. We successfully took the ERC from discovery to GMP manufacture and FDA approval for clinical trials. Clinical production, delivery, and potency assays for ERC are covered in our patent application #61/566460. The use of ERC for treatment of vascular conditions is covered in our patent application # 20090291061, the use for treatment of diabetes was in-licensed to us from Hugh Taylor of Yale University #61/510,812, and the use in traumatic brain injury and Duchenne muscular dystrophy is covered by our patent applications, #61/618974 and #61/164,810, respectively.

Exosomes as Mediators of Paracrine MSC Activity

Exosomes are nanoparticles (40–100 nm) in size that possess highly defined homogeneous characteristics [45]. Exosomes are used by various cells for intercellular communication and have been identified in T cells [46, 47], B cells [48, 49], dendritic cells [50, 51], tumor cells [52, 53], neurons [54, 55], oligodendrocytes [56], and placental cells [57]. Recent studies have demonstrated that stem cell-derived exosomes are responsible, at least in part, for paracrine angiogenic and cardioprotective activity of cell therapy products such as MSC or CD34+ cells [58, 59], given that exosomes can be produced en masse in a bioreactor setting and that safety and distribution of exosomes are conceptually superior to administration of live cells.

Commercial Significance

Development of novel radioprotectants in the area of cell therapy has attracted significant defense interest. Osiris received a $4.2 million upfront grant for large animal studies along with a procurement order of $224.7 million (http://investor.osiris.com/releasedetail.cfm?releaseid=284617), while Cellarant last year received a $153 million award for development and stockpiling of their hematopoietic progenitor cells from the Biomedical Advanced Research and Development Authority (BARDA) for use in radiation sickness (http://www.cellerant.com/pr_090110.html). The "Animal Efficacy" Rule developed by the US Food and Drug Administration (FDA) in 2002 eliminates the requirement for Phase II and Phase III clinical trials for therapies against ARS, since it would be unethical to conduct efficacy studies in humans. In such cases, approval is based upon efficacy studies in representative animal species and only extended Phase I safety, human volunteers. We believe that based on the clinical safety data that will emerge from ongoing trials, we can apply for registration based on animal efficacy experiments, the protocol for which, including dose escalation study and route of administration, will be finalized in Phase II of this project.

Experimental Data

Previous studies demonstrated that the postradiation acceleration of hematopoietic recovery subsequent to BM-MSC administration is mediated by paracrine factors given that the majority of administered BM-MSC are sequestered in the lung [24]. Growth-promoting activities of various stem cells such as CD34 hematopoietic stem/progenitor cells and MSC have been reported to be mediated by exosomes in cardiac infarct recovery model and in hind limb ischemia models [58, 59].

We recently demonstrated that ERC are capable to produce high levels of exosomes. Furthermore, ERC-derived exosomes stimulate proliferation of bone marrow hematopoietic progenitors. Specifically, exosomes were prepared from the supernatant of day 4 ERC or BM-MSC (Cambrex) cultures by differential centrifugation. Conditioned media was subjected to three successive centrifugations at 300 g (5 min),

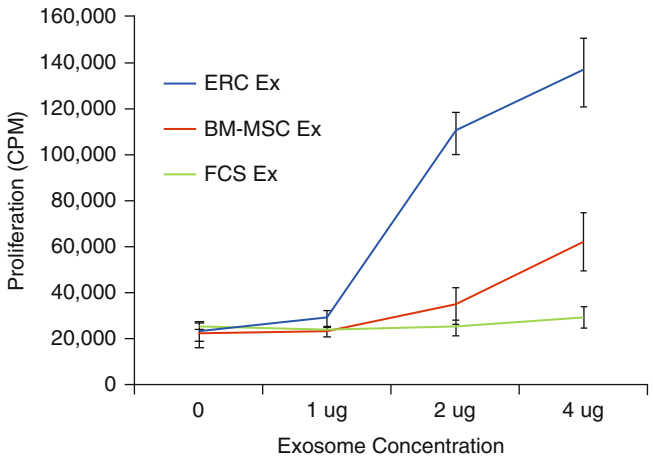

Fig. 4.1 Stimulation of bone marrow mononuclear by ERC and BM-MSC exosomes

1,200 g (20 min), and 10,000 g (30 min) to eliminate cells and debris, followed by centrifugation for 1 h at 100,000 g. To remove excess serum proteins, the exosome pellet was washed with a large volume of PBS, centrifuged at 100,000 g for 1 h, and resuspended in 120 µl of PBS for further studies. The exosomes were quantified by a micro-Bradford protein assay (Bio-Rad). Each batch was standardized by protein content. As a control, we used exosomes isolated from fetal calf serum (FCS Ex). To evaluate stimulatory properties of exosomes on hematopoietic stem/progenitor cell proliferation, mouse bone marrow cells were extracted from femurs and tibia of 6–8-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbour, Maine). Bone marrow mononuclear cells were plated at a concentration of 100,000 cells per well in a volume of 100 ml of complete DMEM media. On day 2, 1 uCi of [3H]thymidine was added to each well 16 h before harvest. Radioactive labeling of proliferating cells was measured on a microplate beta counter (Wallac). Data in Fig. 4.1 demonstrate that human ERC exosomes (ERC Ex) possess a higher stimulatory ability compared to BM-derived exosomes (MB-MSC Ex), which in turn was higher than fetal calf serum-derived exosomes (FCS Ex).

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Endometrial Stem Cells as Potential Cures for Human Diseases

5

Graciela Krikun and Hugh S. Taylor

Introduction

Stem cells are undifferentiated cells that have the ability to self-renew as well as to produce more differentiated daughter cells [1, 2]. Broadly, they can be divided into two categories: embryonic and adult. Embryonic stem cells are found in the inner cell mass of the blastocyst. Adult stem cells, derived from postembryonic cell lineages, have been described in a number of different organ systems and have been best characterized in the hematopoietic system [1, 3]. In addition, because of the limitations in the availability and use of embryonic stem cells, studies began to describe the possibility of using induced pluripotent stem (iPS) cells [4]. iPS cells are adult stem cells that have been genetically manipulated to display properties of embryonic stem cells [5].

Embryonic, induced pluripotent (iPS) and adult stem cells have a vast capacity for differentiation into multiple cell types that may be useful for tissue engineering. The clinical use of embryonic stem cells is limited due to concerns over immune rejection, teratoma formation, availability, and ethical acceptance. The use of iPS adult stem cells is not subject to all of these concerns; however, iPS cells are derived after genetic manipulation; the consequences of these alterations prohibit immediate clinical use pending further characterization [5–7]. Adult stem cells are immunologically identical to the individual from whom they are obtained.

Adult stem cells have a vast capacity for differentiation into multiple cell types that may be useful for tissue engineering [8–19]. Adult stem cells, present in normal adult tissue, have great plasticity (they can differentiate into cells different from those in tissues from which they were taken) and can proliferate in vitro [20] and are immunologically identical to the individual from whom they are obtained [21]. Further, they can often be differentiated using specific culture techniques and defined media without transfection. These characteristics have already allowed the use of adult stem cells in several clinical applications [22, 23]. Currently, it is known that almost all tissues of an adult organism have somatic stem cells [24]. Although the differentiating potential of embryonic stem cells is higher than somatic stem cells (SSCs), several studies suggest that some types of SSC, such as mesenchymal stem cells (MSCs), can be induced epigenetically to differentiate into tissue-specific cells of different lineages [24]. This unexpected pluripotency and the variety of sources from which they can be derived can make MSCs suitable for the treatment of diverse pathologies. In this review, we will focus on MSCs derived from the endometrium, a constant renewable source of MSCs even past menopause.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) can be defined as multipotent stem cells which hold the ability to self-renew and differentiate into multiple cell types including osteoblasts, chondrocytes, adipocytes, endothelial cells, myocytes, and others [25–29].

MSCs can be easily isolated from several tissues and generally form fibroblast-like, plastic-adherent cells that form colonies in vitro [25, 30]. Since their original description in 1974 [30], MSCs have received a lot of attention in regenerative medicine due to easy isolation, low immunogenicity, multi-differentiation potential, immunomodulatory properties, and lack of ethical controversy [25].

One critical aspect of MSCs is their ability to hone in on damaged tissues and migrate to the injured site [31]. MSCs used in several studies were isolated from different tissues, including the bone marrow, umbilical cord, adipose tissue,

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and placenta, and, in our studies, from the endometrium [32–41]. However, the precise differences of migratory capacity between different sources of MSCs have not been fully defined and we predict differences will be detected depending on the source of origin [25, 31].

Although MSCs can differentiate into various mature cell phenotypes, perhaps more important is their intrinsic capacity to secrete cytokines and growth factors at sites of tissue injury and inflammation, contributing significantly to their therapeutic capacity [42].

Cell therapy offers enormous hope for solving some of the most tragic illnesses and tissue defects; however, a significant barrier to the effective implementation of cell therapies is the inability to target a large quantity of viable cells with high efficiency to tissues of interest [42]. Thus, MSCs represent a potential source of immunoprivileged postnatal cells that are conveniently isolated autologously or used from an allogenic source without the addition of an immunosuppressive regimen [42]. The mobilization and engraftment of stem cells have enormous significance for tissue repair and regeneration as well as to our understanding of the processes of aging and disease.

Endometrial-Derived Stem Cells

The uterine endometrium is one of the most dynamic human tissues and consists of a glandular epithelium and stroma that are completely renewed in each monthly menstrual cycle. Endometrial stem cells are thought to reside in the basalis layer and serve as a source of cells that differentiate to form the endometrium. These endogenous stem cells allow the rapid regeneration of the endometrium necessary to support pregnancy [43]. We proposed that these stem cells were bone marrow derived. In order to prove this, we studied four female allogenic bone marrow transplant recipients who received marrow from a single-antigen mismatched related donor and therefore had an HLA type that allowed determination of the origin of any cell. To avoid the possibility that men may not harbor female reproductive tract stem cells, each recipient had received marrow from female donors. All bone marrow transplants were performed for leukemia treatment. All women were of reproductive age and had received total body irradiation and chemotherapy with cyclophosphamide and in two cases also cytarabine or busulfan at least 2 years before biopsy. HLA type was determined by immunohistochemistry and by reverse transcription–polymerase chain reaction.

Donor-derived endometrial cells were detected in endometrial biopsy samples from all bone marrow recipients and accounted for 0.2–48 % of epithelial cells and 0.3–52 % of stromal cells. None of the controls demonstrated HLA mismatch in endometrial samples. Our findings demonstrated that endometrial cells can originate from donor-derived bone marrow cells and suggest that non-uterine stem cells contribute to the regeneration of endometrial tissue [43].

In 2007, two studies showed the existence of a small population of multipotent cells in the endometrium [44–47]. It was demonstrated that endometrial MSCs could be isolated by the co-expression of two perivascular cell markers, CD146 and PDGF receptor-β (PDGF-Rβ) [44]. It was the first time that markers were used to identify these mesenchymal stem cells. The CD146+PDGF-Rβ+ cells were found in the perivascular region in both functionalis and basalis layer of the endometrium. These cells expressed typical MSC surface markers (CD29, CD44, CD73, CD90, and CD105) and were negative for hematopoietic and endothelial markers and also underwent differentiation into adipogenic, myogenic, chondrogenic, and osteoblastic lineages when cultured in appropriate induction media.

Studies from our laboratory have similarly showed that the uterine endometrium contains multipotent stem cells that have the ability to proliferate in culture, express a consistent set of marker proteins on their surface, and can be reproducibly differentiated to multiple non-endometrial cell lineages under controlled in vitro conditions [44, 47–56]. These cells are a particularly appealing source of mesenchymal stem cells as the endometrium is readily available due to continuous regeneration in reproductive aged women and can easily be restored with estrogen treatment after menopause.

Endometrial cells are readily obtainable by endometrial biopsy, a less invasive and less painful procedure than bone marrow biopsy. Bone marrow aspiration is typically performed under general anesthesia, while endometrial biopsy is a simple office procedure. In women, the endometrium provides a source of immunologically matched cells for tissue engineering without concern for rejection. Additionally there are approximately 600,000 hysterectomies performed yearly in the United States alone that could serve as a donor bank providing stem cells for men and for women [57, 58].

We have shown that human endometrial-derived mesenchymal stem cells (hEMSCs) have the ability to treat human diseases including type 1 diabetes and Parkinson's disease [21, 48].

Insulin Producing Cells Derived from hEMSCs to Treat Diabetes in a Murine Model

Diabetes is a chronic metabolic disease in which the insulin-producing β-cells of the islets of Langerhans are destroyed [59]. While transplantation has been one of the techniques that led to much hope, it is difficult to find adequate donors and the procedure involves surgical intervention to the donor and the recipient. One method to overcome donor–host rejection in the treatment of type 1 diabetes is autologous or semi-allogenic stem cell transplantation. Our studies have shown that hEMSCs can be reprogrammed into insulin-producing

cells [21]. We found that only cultures that were treated with modified protocols described by others [60] and optimized in our laboratory [21] displayed clear insulin production [60]. Reverse transcription–PCR (RT-PCR) demonstrated that the differentiated cells had increased expression of pancreatic lineage markers compared with the undifferentiated cells. Levels of PDX1, PAX4, GLUT2, and insulin (INS) mRNA all increased significantly in the treated cells ($p<0.001$). The expression of PAX4 confirmed that our protocol favored the β-cell lineage fate. Moreover, immunohistochemical studies showed that insulin was not detected in undifferentiated cultures, whereas a strong signal was found in the clusters of differentiated cells [21].

To assess the potential use of these cells for therapeutic purposes in patients with type 1 diabetes, we tested their effectiveness in a model of diabetes using severe combined immunodeficiency mice. The destruction of the pancreatic β-cells was achieved by treatment with streptozotocin (STZ). Mice with blood glucose measurement levels above 220 mg/dl in response to STZ treatment were chosen as subjects for the experiment. The control group consisted of animals treated with STZ that received transplants of undifferentiated cells. In the latter, glucose levels increased significantly to a peak by the end of the fourth week after the transplant. By contrast our experimental group that was treated with differentiated hEMSCs showed a stabilization of glucose [21].

Mice from the group that were transplanted with differentiated cells displayed no gross pathological symptoms. In contrast, diabetic mice that were either not transplanted or transplanted with undifferentiated hEMSCs showed a large number of complications in the initial observation period including cataracts, obvious signs of dehydration, loss of skin resiliency and prolonged recovery time after pinching, loss of fur sheen, and passive, sedate behavior [21].

Hence, the endometrium offers an ideal source of MSCs that can be differentiated towards insulin-producing cells and may be a critical resource for patients with type 1 diabetes.

The Use of Differentiated hEMSCs to Treat Parkinson's Disease in a Murine Model

Parkinson's disease (PD) is a neurodegenerative disorder caused by the loss of dopaminergic neurons [61, 62]. As with other serious diseases requiring intervention, one method to overcome donor–host rejection in the treatment of Parkinson's is again either autologous or semiallogenic stem cell transplantation.

Recent studies from our laboratory have shown that hEMSCs can be reprogrammed into dopaminergic producing neurons [48].

Hence, in addition to morphological and immunostaining characteristics, in vitro differentiated cells expressed electrophysiological properties of neurons. A whole cell patch clamp recording method was used to measure the current characteristics of individual cells to look for evidence of barium-sensitive potassium channels, which are characteristic of central neurons, including dopaminergic cells [48]. The cells exhibited neurogenic morphology including long axon projections, pyramidal cell bodies, and expression of tyrosine hydroxylase (TH), the key enzyme involved in dopamine production [48].

The discovery of -methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a selective neurotoxin of dopaminergic cells is an engrossing story of a chemistry student trying to produce heroin, but failing to do so produced MPTP instead which resulted in severe cases of Parkinson's disease in drug users [63, 64]. The drug induces Parkinson's in both animals and human beings.

Thus, both immunodeficient and immunocompetent MPTP lesioned mice were successfully transplanted with differentiated hEMSC. Engraftment was demonstrated up to 5 weeks following transplantation [48]. Transplanted mice were found to express human TH by RT-PCR, whereas sham-transplanted animals did not. Intracranial transplantation with hEMSC resulted in a significant improvement of striatal dopamine and dihydroxyphenylacetic acid concentrations in this Parkinson's mouse model.

Discussion

Contemporary approaches to cure life-threatening diseases such as type 1 diabetes or Parkinson's disease have had negligible success. One promising development has been the findings that stem cells can be directed to differentiate into specific cell types.

Adult stem cells are not subject to clinical or ethical barriers as opposed to embryonic or iPS cells [65–69].

The endometrium is a tissue that is continuously regenerated and we have now shown that it contains bone marrow-derived stem cells. In addition resident stem cells can be isolated and differentiated into insulin-producing cells or L-DOPA-producing cells. As the endometrium is readily available, easily obtainable tissue which can be attained in an uncomplicated office procedure, it makes the endometrium a particularly attractive source of adult stem cells. Based on the therapeutic potential we demonstrate here that hEMSC may become an important source of allogenic stem cells to be used for regenerative medicine, we continue our studies to understand the pathways leading to these promising outcomes.

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Menstrual Blood Transplantation Therapy for Stroke and Other Neurological Disorders

6

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Introduction

Cell-based therapy has evolved over the years as a robust experimental treatment for regenerative medicine, but the extension of its potential entry into clinical application is still being explored. Bone marrow transplantation is one of the first and most frequently studied applications of stem cells, and replacement of diseased bone marrow by healthy cells has improved survival of thousands of patients. Following the knowledge provided by hematopoietic stem cell transplantation, other cell sources have been investigated, including embryonic, fetal, and adult tissues. In addition, different applications for stem cell therapy have been studied, not only aiming replacement but also targeting inflammation and tissue repair.

Several diseases are marked by inflammatory responses, either as etiopathogenic events or as a consequence of disease progression. In stroke, for instance, the inflammatory reaction that follows ischemia may increase the injured area to limits beyond those caused by the ischemic insult itself, providing an opportunity to a much far-ranging therapeutic application of stem cells. These cells have the ability to modulate the immune system, establishing a less inflammatory environment, increasing immunological tolerance, and decreasing cell death. To date, studies have investigated umbilical cord blood cells, neural cells, mesenchymal cells from diverse tissues, and adult hematopoietic stem cells as sources for the treatment of stroke, all reporting success to some extent. However, most investigations are restricted to animal studies, as clinical translation is sometimes difficult. Less invasive forms of cell

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administration, use of cells that are better tolerated by the host immune system, and availability in easily accessible tissues are priorities to most recent research. Moreover, the use of adult stem cells as primary donor source for cell therapy has become a topic of contention in both the scientific field and the public community because of safety and ethical concerns that hinder clinical applications of cells from embryonic and fetal origins.

Knowledge about the mechanisms of action of the cells on the injured site has changed over the years, contributing to advances in their application. Firstly, cell differentiation may not be necessary to promote repair. This observation implies that cells derived from adult tissues, which do not usually differentiate as easily as the pluripotential cells from more immature tissues, may be used. Second, regeneration may occur without cell migration to the site of injury. This finding implies the utility of intravenous applications of the cells, instead of the more invasive local injections. As an extension to this idea is the notion that cell therapy for central nervous system disorders may proceed without the need for mechanically disrupting the brain (i.e., neurosurgical manipulations), since cells no further need to cross the blood-brain barrier to reach their therapeutic target.

Today, it is established that stem cells may be derived from numerous tissues, with some variability in their therapeutic potentials. In this context, menstrual cells may be a precious finding, because they derive from a disposable tissue, are readily available, and share common properties with less mature cell types, such as high differentiation potentiality and little immunogenicity. A major advantage of menstrual cells is their application for autologous transplantation which should circumvent many immunological complications and adverse side effects associated with mismatched donor cells and transplant recipients. Recently, these cells have been applied in experimental and clinical studies, and the effect of menstrual blood cells on basal ganglia disorders is the main subject of this chapter.

Investigation of Stem Cells

Today's challenge is to find the perfect cell, which should be immature enough to hold multipotential differentiation properties and yet safe to not induce malignancy. It should also modulate the immune system, decreasing destructive aggression but preserving its ability to fight pathogens. Finally, it should be able to induce changes in the targeted tissue, either restoring its function or promoting repair. Several cell types match the above criteria and have been applied in experimental and clinical research; however, in most cases ethical and practical issues are a concern. Stem cells from bone marrow, for instance, work well on most studies, but cell harvesting through bone marrow aspiration or leukapheresis is needed and the number of cells obtained may be not enough. Other sources, like the liver, skin, heart, or even induced pluripotent stem (iPS) cells, are also available but the isolation and culture of those cells is currently costly and technically complicated [32, 42, 65]. There is interest, therefore, in acquiring stem cells from disposable and easily accessible tissues, such as the amnion and amniotic fluid, placenta, umbilical cord tissue, adipose tissue, and, more recently, menstrual blood.

Because of ethical and safety reasons associated with embryonic stem cells, the last decade has witnessed the wide use of adult stem cells as graft source for cell therapy. In the United States, the previous government moratorium on the use of embryonic stem cells also arguably influenced the shift of cell based-therapies toward the use of adult stem cells. A majority of the studies have explored adult stem cells harvested from the bone marrow. Nonetheless, although numerous applications of hematopoietic stem cell transplantation for malignant and nonmalignant diseases have been reported, there is still a paucity of this treatment in neurological disorders. Moreover, compatibility issues restrict the use of adult hematopoietic stem cells, requiring fully or close to fully HLA-matched donors. In the last years, new adult stem cell donor tissues have been investigated, as alternatives to bone marrow, comprising a rich research field to be explored. Umbilical cord blood, amnion, and placenta provide stem cells that are thought to possess a higher immunosuppressive potential, are better tolerated by the host immune system, and preserve multipotential differentiation capacity. Of note, the mesenchymal cells, or stromal cells, harvested from those tissues share interesting phenotypic and functional features with embryonic stem cells and also to mesenchymal cells derived from bone marrow, like the ability to modulate the immune system and to stimulate tissue repair, which have already been applied for the treatment of autoimmune and degenerative diseases.

Stroke

Stroke is the third leading cause of death [86] and the primary cause of long-term disability in the United States [18]. Despite successful efforts to decrease incidence and mortality of cerebrovascular diseases, they still remain as a major concern in clinical setting. There is a negative impact on economic productivity, as approximately 15–30 % of first-time stroke patients become permanently disabled and 20 % still require institutional care 3 months after stroke [7]. For the year of 2010, the American Heart Association estimated direct and indirect costs of stroke of $73.7 billion [54]. Therefore, every effort to decrease the incidence of strokes and their consequential sequelae is urgently warranted.

Tissue plasminogen activator (tPA) is the only therapeutic agent approved by the US Food and Drug

Administration indicated for ischemic stroke. The drug catalyzes the transformation of plasminogen into plasmin, which acts as a potent thrombolytic agent and is used to restore the blood flow, thus minimizing immediate tissue death. In 1995, an American clinical trial demonstrated that patients treated with tPA within 3 hours of beginning of symptoms presented less disability 3 months later [81]. The results were also supported by a similar concomitant European trial [36]. Since then, the FDA and, later, the European Medicines Agency have approved the drug, and it has been applied as standard therapy in emergency rooms all over the world. Numerous other studies also supported the early use of tPA, demonstrating a direct correlation between time elapsed to begin treatment and long-term neurological impairment [1, 59, 71].

The application of tPA, however, is greatly limited by the therapeutic window of 3 h. Most patients are not able to reach an Emergency Room and complete the neurologic triage within that timeframe and, therefore, are excluded from the beneficial effects of the drug. In fact, in 2008 only 1.8–2.1 % of all patients affected by ischemic strokes in the United States received the therapy [46]. Further studies have tried to evaluate the possibility of extending the limit beyond 3 h, but the results were nonconclusive, with an increase in mortality due to hemorrhagic complications [17, 22, 37]. Some controversy was created by subsequent studies, which showed that the results of tPA treatment were not as favorable as once suggested and, most importantly, that its beneficial effects were restricted to a small population of the stroke patients. In 2006, analysis of the European data indicated that higher age; male gender; history of previous strokes; comorbidities, such as hypertension and diabetes; and higher severity of the stroke were associated with poor outcomes after tPA therapy [44]. Moreover, new research has shown that while tPA renders clot clearance within the intravascular space, it can be deleterious to the brain through worsening of the already altered blood-brain-barrier permeability, in addition to direct neurotoxic effects [87]. It is clear that tPA for ischemic stroke benefits a very selective group of patients. Those who are not able to avail of the 3-h tPA efficacy period and would have to deal with the long-term consequences of the disease, most often, are relegated to rehabilitation therapy. This significant unmet clinical need for stroke thus warrants investigations into novel treatments with wide therapeutic windows such as restorative interventions, including cell-based therapy.

Inflammation After Stroke

The acute blood supply interruption that takes place in ischemic stroke promotes almost instant cell death of the infarct core. The surrounding ischemic penumbra area comprises the tissue that retains structural integrity, but lacks function. It has a less defined outcome and may either evolve to death or to recovery, depending on the reestablishment of blood flow within the first hours after stroke [34]. Once the hypoxic insult is settled, a complex cascade of events takes place, in which inflammation seems to have a major role [26]. Following the sudden drop in oxygen and glucose supply that begins immediately after the interruption of blood flow, intracellular Ca^{++} concentrations increase, as a consequence of the Na^{+}/K^{+}-ATPase and Ca^{++}/H^{+}-ATPase pumps malfunctioning, associated to the reversed function of the Na^{+}/Ca^{++} transporters [25]. The high concentrations of the ions activate Ca^{++}-dependent enzymes, leading to cytotoxicity, formation of free radicals, and cell death. In parallel, ischemia triggers neuronal depolarization, leading to glutamate excitotoxicity, considered a major pathophysiological event of stroke [69], also contributing to neuronal death. Soon other inflammatory responses arise, with generation of free oxygen radicals and nitric oxide, activation of neighboring glia, and migration of other inflammatory cells from the systemic circulation, which once more promote further neuronal death.

Local and migrated cells participate of the inflammatory response. Activated astrocytes contribute to the formation of glial scar, therefore limiting the extension of injury. In long term, however, the scar restrains the migration of cells and hampers repair of the injured tissue. Interestingly, Faulkner et al. [29] demonstrated that the inhibition of astrocyte activation following spinal cord injury increased neuron death, possibly because astrocytes also secrete neurotrophic factors (NGF and BDGF), which are important for tissue repair and modulation of synaptic plasticity [47]. The microglia also take part in recovery by removing debris and harmful substances from the parenchyma. Likewise, neurons themselves contribute to increase the inflammatory response, through the release of free radicals and inflammatory cytokines. Finally, endothelial cells are also affected, with opening of the blood-brain barrier and subsequent passage of cells and proinflammatory agents into the central nervous system (CNS).

In the last years, research has targeted inflammatory components of stroke, aiming to reduce the extension of injury in the CNS. Anti-inflammatory interventions may attenuate the secondary cell death associated with ischemic stroke and, likely, lessen neurological impairments among other progressive disabilities. Specific inflammatory agents have been blocked, such as ICAM-1, IL-1 receptor, nitric oxide, TNF-α, and, more recently, Toll-like receptors [27]. Others have focused on more general interventions, also trying to abrogate inflammation, such as induced hypothermia, corticosteroids, and minocycline [28, 50, 60]. To this end, finding a novel anti-inflammatory strategy, such as the use of cell therapy, to at least minimize the injuries and at most reverse the outcome of stroke is a logical therapeutic approach toward improving the quality of life for many stroke patients.

In the cell-based therapy context, inflammation is necessary. The presence of cytokines is essential for cell migration to the site of injury and for production of neurotrophic agents, but their excess may decrease graft survival and directly contribute to neuronal death. On the opposite way, the stem cells themselves may modulate the inflammatory setting, inhibiting the reactive immune cells, increasing tolerance, and promoting a cytokine shift toward a less inflammatory Th2 profile [45, 53, 75].

Injury following stroke can be divided in three consecutive stages, important for the different therapeutic opportunities implied in each [40]. In the 24 h that immediately follow stroke, attempts to restore the blood flow would be neuroprotective, therefore preventing further early neuronal death and restricting the extent of the penumbra area. Thereafter, once the injury is established, the interventions would be mainly restorative and cell-based therapies would have their best indications. It is known that during the first month after stroke the brain produces inflammatory signals, which can be used to opportunely attract cells injected in the systemic circulation to the site of injury. It is also during this period that the interventions may restore the viability of the tissue in the penumbra area. Terminated the first month, the inflammation decreases and scars and structural damage persist. Stem cells still have a possible therapeutic role in this last phase, but they should be delivered directly into the nervous tissue, through the aid of scaffolds and surgical procedures.

Inflammation plays a double-edged sword role in stroke, as in other CNS inflammatory and neurodegenerative diseases [3, 23, 56]. Contrary to previous beliefs, it is unquestionable today that inflammation and immune activation are present in the CNS and, even further, that the CNS influences and is influenced by systemic inflammatory processes. Following stroke, inflammatory markers, such as IL-6, C-reactive protein, matrix metalloproteinase, and monocyte chemoattractant factor-1 (MCP-1), are detected in increased levels in the peripheral blood [85]. In the brain tissue, cytokine production is induced immediately after the ischemic injury, followed by the release of chemokines and adhesion molecules, recruitment of immune cells to the parenchyma, and, finally, activation of immigrated and endogenous cells [72, 90]. Some of the inflammatory agents, such as IL-6, nitric oxide (NO), and TNF-α, have antagonistic effects on the tissue, acting as protective in early phases and detrimental in late stages of injury [57, 62, 78]. For instance, in multiple sclerosis, which is an inflammatory autoimmune disease, TNF-α is known to mediate injury, but blocking this cytokine in a clinical trial resulted in worsening of the disease [80]. Shohami et al. [76], on the other hand, demonstrated that the inhibition of TNF-α improved the outcome of closed head injury rats, decreasing inflammation and protecting neurons from death. Based on such observations, it is discussed that the timing of intervention, either stimulating or inhibiting inflammatory responses, is of main importance for the success of treatment [77].

In summary, at early stages, inflammation is desired and necessary. However, at chronic period, if inflammation persists and no therapeutic intervention is employed to correct this aberrant response, then such host tissue response to injury will exacerbate the disease progression. Along this line, the initiation of anti-inflammatory regimen after stroke is critical to the resulting outcome, and it represents an opportune niche for the application of cell-based therapies. Additional critical analyses of inflammation as a friend and foe of stroke are needed to fully decipher the mechanisms of action of inflammation in stroke onset and progression.

The Role of Stem Cells in Stroke

In stroke, inflammation must be restrained and tissue destruction must be minimized or even repaired. Reinforced by the previous knowledge acquired from treatment of basal ganglia disorders, stem cells seem the ideal therapeutic tool. To date, several studies using stem cells for experimental stroke have been published [15, 30, 48], and their beneficial effects are becoming well established, although still questioned by some researchers [88].

The use of stem cells in stroke is criticized because their exact targets and mechanisms of action are still unknown and need investigation. However, evidence shows that they can be effective through multiple pathways, all leading to improvement of the injury. Taguchi et al. suggested an angiogenic effect of CD34-positive (CD34+) cells from umbilical cord blood on the ischemic area of stroke [79]. They observed that the cells injected systemically into a mouse model of stroke secreted growth factors (VEGF, FGF2, and IGF-1), induced formation of vascular channels, and, secondarily, promoted migration of neuronal precursors into the injured areas, which differentiated and improved nervous function. The addition of anti-angiogenic agents abolished the beneficial effect of the cells, demonstrating the importance of vessels in nervous repair. The issue was later discussed by Saghatelyan [74], which supported the hypothesis that vasculature-guided neuronal migration can be observed not only following stroke but also as part of the normal brain development. More recently, endothelial progenitor cells injected into the systemic circulation of mice migrated to the stroke area, promoted repair, and improved behavior, in comparison to controls, reinforcing the importance of angiogenesis [91]. Finally, Nakagomi et al. [66] demonstrated that the addition of endothelial precursors to neural stem cells, engrafted in mouse models of brain ischemia, enhanced cell survival, proliferation, and differentiation, when compared to injections of neural stem cells alone.

In the same study published by Taguchi et al., the authors reported that CD34-negative (CD34-) cells were not able to promote tissue repair after stroke. Boltze et al. refuted this last idea through a study in which CD34+ and CD34- cell populations from umbilical cord blood were injected intravenously and had equivalent effects on rat models of stroke [12]. In particular, both cell subtypes promoted functional improvement and migrated to the area of injury, although differentiation into neural markers expressing cells was not detected. The authors hypothesize that CD133+ CD34- cells might have a role in tissue repair equally as CD34+ cells. This type of discrepant results, although may be construed as hindering the progress of cell therapy, actually exemplifies that the field is maturing and that there is a healthy atmosphere among stem cell researchers to confirm, refute, and validate efficacy and safety data. In the end, such rigorous preclinical studies will benefit the transplant recipient.

Until recently, it was believed that cell effectiveness would be conditional on their migration to the site of injury. In fact, several authors observed a direct relationship between cell migration to the site of injury and behavioral improvement [8, 43]. However, Borlongan et al. [15] observed, in rat models of stroke, that umbilical cord blood cells were able to promote repair even when not detected in the tissue, probably through the production of growth factors, cytokines, and other therapeutic molecules that were able to reach the target. Adding importance to that idea, in the last years, considerable research has studied the effect of neurotrophic agents on stroke, as it happened in basal ganglia disorders. Neurotrophic agents influence cell survival, proliferation, differentiation, function, and plasticity [39, 55]. They also have a role in physiological endogenous repair, and increased levels can be detected in injured neuronal sites [21]. They protect neurons from the cytotoxic insults generated during inflammation, with anti-excitotoxic and antioxidant functions, besides improving mitochondrial function.

Administration of agents with specific angiogenic functions, such as VEGF, angiopoietins, factors that influence Notch signaling, among others, have generated interesting results in neural support [2, 4, 5]. Additionally, some studies have addressed the use of genetically modified cells to release neurotrophic factors at the site of injury. The cells would be used as vehicles, attracted to the inflammatory area of stroke, and the growth factors would promote angiogenesis and provide support to neuronal progenitors in the brain [61]. Neurotrophic agents are considered promising for stroke therapy, but their stable and long-term effectiveness still warrants additional investigations, owing in part technical problems related to the manufacturing production of these molecules at clinically therapeutic doses. The combination of neurotrophic factor treatment with cell therapy seems interesting and perhaps will provide exciting results in the future.

Parkinson's and Huntington's Disease

Experimental stroke studies share overlapping research themes with other animal models of basal ganglia disorders, mainly Parkinson's disease and Huntington's disease, in which numerous therapeutic strategies have been described, aiming to improve survival of remaining neurons, abrogate the ongoing neurotoxic processes, or functionally replace the destroyed tissue [2]. Cell-based therapy is considered promising and different cell types have been used. Fetal neural tissue, neuronal stem and progenitor cells, tissue engineered to secrete neurotransmitters or neurotrophic factors, paraneuronal cells which support neurons, and grafts that may assist the reconstruction of injured axonal pathways have been documented in the literature, with variable results. A major long-standing conundrum in cell therapy is finding the most optimal donor cells. While the more differentiated cells have been thought to offer tissue-specific transplant regimen tailored to treat the specific diseases, their mature phenotype (albeit also mature immune system) renders them to be immunologically rejected by the host and, therefore, rapidly eliminated. On the other hand, less differentiated cells such as the embryonic stem cells are also attractive, since they are better tolerated by the host immune system and have more plasticity, but the risk of uncontrolled proliferation and tumor formation strongly limits their applications.

Parkinson's disease is a common neurodegenerative disease that affects about 2 % of the population over 65 years of age. Progressive loss of the dopamine-producing neurons from the midbrain substantia nigra, that project into the striatum, cause tremor, rigidity, and hypokinesia. Experimental studies have been conducted for approximately 30 years, with interesting data from cell-based therapies, despite some major hurdles [10, 11, 33]. The best results were achieved with transplantation of fetal mesencephalic tissue into the striatum, which is the target for dopamine action. In humans, transplantation for Parkinson's disease has been reported since 1982, with initially successful and later disappointing results [9, 51, 58]. The major barrier observed was poor survival of adult grafts in the host brain, which was to some extent solved with the use of tissue from fetal origin. Still, only about 10 % of the transplanted cells survive in the host tissue [38]. Fetal mesencephalic tissue has been used to replace the lost neurons and restore dopamine production and comprises an important progress in the field. To date, between 300 and 400 patients have already been grafted with the fetal mesencephalic tissue, but while some report outstanding outcomes, with some patients leaving medication, others fail to observe major effects [6]. The inconsistency of results, ascribed to ethical concerns, limited tissue availability, emergence of unexpected dyskinesias, and high rates of graft rejection, still hamper the application of this therapy and stimulate the search for alternatives.

Huntington's disease is genetically determined, with progressive neurodegeneration primarily of the striatum, inducing motor and psychiatric dysfunctions. As in Parkinson's disease, several attempts to delay the progression of the disease have been studied. Treatment of Huntington's disease, though, is complex and requires more than simple neurotransmitter replacement, in that reestablishment of the striatal network is needed. Transplantation of fetal striatal tissue proved to be effective in experimental models of the disease, with symptomatic improvement and evidence of graft incorporation; however controversial establishment of connections with the host striatum remains to be resolved [13, 14, 35]. Clinical trials have been conducted with modest results; while some patients present motor and cognitive improvements after transplantation, others fail to benefit from the procedure [52].

In evaluating the progress of cell therapy for Parkinson's and Huntington's diseases, it is evident that there are technical disparities among the centers that might influence the results and some guidelines are to be determined. Age of the patient, disease stage, number of cells, surgical technique to implant the cells, and tissue conservation, among others, are reported as parameters to be defined. Moreover, neurotrophic factors promote neuroprotection, which is essential in both diseases, and some authors propose to combine their use with the transplanted cells [2]. Other strategies, using antioxidant, anti-excitotoxic, bioenergetic, immunosuppressants, and antiapoptotic agents are also suggested either as stand-alone monotherapy or as adjunct to cell transplantation.

At present, besides the satisfactory results obtained with the transplanted fetal tissue, there is a continued search for other cell sources aiming to treat Parkinson's and Huntington's diseases. Little availability of the embryonic tissue is the main reason. Recently, a clinical trial conducted in India tested mesenchymal cells for Parkinson's disease, but while safety was established, efficacy could not be evaluated due to the study design [82]. Similarly, rat models of Huntington's disease were treated with mesenchymal cells genetically modified to express growth factors, evolving with delay of disease progression [24]. Other researchers focus on neural tissue differentiated from human embryonic stem cells, but the studies are still in the experimental level, mainly because of safety reasons. These cells, even after differentiation, retain the capacity to form tumors, and attempts to reverse it have decreased their survival [31, 73]. Finally, there is recent effort in treating Parkinson's disease with autologous induced pluripotent stem (iPS) cells, which are interesting for their adult cell origin, avoiding ethical issues. Their safety, however, remains questionable, since formation of neural overgrowths was observed in rats transplanted with these cells, similarly to what was seen with embryonic stem cells [83], likely owing to the lack of regulatory mechanisms in controlling the "stemness" technology employed to revert these mature cells into their undifferentiated plastic state.

Several cell-based studies are being investigated for basal ganglia disorders, mainly using cells already differentiated into neural tissue. While it is still early to apply them in large-scale clinical trials, much of the knowledge acquired from experimental studies can be transposed to the treatment of other diseases. Stroke, for instance, is also considered a basal ganglia disease, and cell therapy has been extensively applied to it as discussed above.

Applications of Stem Cells Derived from the Endometrium

The presence of stem cells in the endometrium was described about 30 years ago, from the observation that the upper layers of this tissue shed and were renovated each month, but that the cells that were shed represented nonviable cells. In particular, the cells in the basalis were viable but not the shed cells from the functionalis [67, 70]. Only in the last few years, however, have endometrial cells been better characterized [19, 63, 68]. In vitro investigations have determined clonogenicity, multipotentiality, and expression of markers of pluripotency, such as Oct-4, SSEA-4, and c-kit, which are frequently found in more immature cell types, including the embryonic stem cells. Finally, an experimental study demonstrated that menstrual blood-derived stromal cells promoted functional improvement of damaged heart tissue, with evidence of cell engraftment and transdifferentiation into cardiac tissue [41].

In the nervous system context, Borlongan et al. [16], published the results of menstrual blood cell transplantation in experimental stroke. Stromal-like menstrual blood stem cells were isolated, expanded, and, at last, selected for CD117 or C-kit receptor, a marker associated with high proliferation, migration, and survival [20]. In vitro studies showed that the expanded cells maintained expression of embryonic-like stem cell phenotypic markers, such as Oct4, SSEA, and Nanog, even when cultured up to 9 passages, as an evidence of the safety and reliability of these cells, and some were induced to express neural markers (MAP2 and Nestin) [16, 68]. Moreover, when added to cultured rat neurons exposed to a hypoxic insult, the menstrual blood cells provided neuroprotection and when applied to rat stroke models, less neurologic deficit was observed on behavioral tests, irrespective of the injection site, i.e., systemic or local administration into the striatum. However, analysis of the tissue, after animal sacrifice, revealed that although human cells were detected in the rat brain, some migrating to areas other than the injected, they did not show signs of differentiation, expressing their original markers. Once more, there is evidence that cell differentiation is not the main pathway of neuroprotection or neuroregeneration.

Wolff et al. [84] reported the use of endometrial-derived neural cells in a Parkinson's disease immunocompetent mouse model. Endometrial-derived stromal cells were differentiated in vitro into dopamine-producing cells, which expressed the neural markers nestin and tyrosine hydroxylase, and were then engrafted into the brains of the animals. Migration, differentiation, and production of dopamine were detected in vivo, demonstrating the therapeutic potential of these cells to functionally restore the damaged tissue, either through cell replacement or endogenous repair.

The only clinical study yet published evaluated the safety aspects of endometrial-derived stromal cells administration [89]. Four patients with multiple sclerosis were treated with intrathecal injections of 16–30 million cells and one of the patients also received an additional intravenous injection of the same cells. No adverse events were registered, as expected, and the authors reported functional stabilization. However, the longest follow-up reached 12 months, and any conclusions about effectiveness of the treatment seem premature in this long-term and slowly progressive illness.

Future Expectations

Research on cell therapy for stroke has reached great proportions, especially because of the possibility of translational studies, which have already started. Most studies use the knowledge of neuroregenerative areas of the brain, more specifically the hippocampus and, still with some controversy, the subventricular zone to guide their studies, although some have shown cell migration and repair of areas other than those considered as highly neurogenic [49, 64]. Furthermore, it seems clear that the rescue of the penumbra area after stroke is decisive for functional outcome and a great opportunity for cell therapy. Stem cells promote neuroprotection, via modulation of the activated immune system as one major pathway. Tissue repair is also described and, although cell differentiation is observed in the experimental setting, its importance to the final outcome of the treatment is still undefined.

Menstrual cells are a novel therapeutic option in this field and have great potential. Ease of access, availability, and safety are considered their main key to future clinical studies. Most important for effectiveness purposes, however, is the immature behavior of these cells, in which pluripotency and proliferation circumvent the main challenges of tissue repair. The menstrual cells represent, therefore, an important therapeutic tool that may improve the outcome of stroke and decrease the disability of future patients. Further experimental studies are still required, especially to determine the exact mechanisms of action and, perhaps, establish new therapeutic applications for these cells.

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Characteristics and Therapeutic Potential of Menstrual Blood-Derived Stem Cells

7

Nurjannah Achmad and Martin Götte

Adult Stem Cells in the Human Endometrium

The Human Endometrium During the Menstrual Cycle: Physiology of a Highly Regenerative Tissue

The human endometrium forms the inner lining of the uterus. Originating from the Müllerian ducts, it is comprised of two major zones in the adult organism (Fig. 7.1): The functional layer (functionalis) largely contains glands extending from the luminal surface columnar epithelium as well as a supportive stroma. The basal layer (basalis) is composed of the basal region of the glands, a comparatively dense stroma, lymphoid aggregates, and blood vessels, including the characteristic spiral arteries in the luteal phase of the menstrual cycle. Furthermore, the endometrial stroma of both layers is populated by different classes of leukocytes, including the tissue-specific uterine natural killer cells, mast cells, macrophages, T and B cells, and neutrophils [1].

During the reproductive years of women, the endometrium is a highly regenerative tissue and a remarkable example of controlled tissue remodeling governed by cyclic endocrine changes [2]. During reproductive life, the human endometrium can undergo about 480 cycles of growth, breakdown, and regeneration. During one menstrual cycle, the endometrium grows from 0.5–1 to 5–7 mm in thickness [3], demonstrating an enormous regenerative capacity. The functional layer of the endometrium is shed about every 28 days during menstruation, which lasts between 3 and 5 days and yields on average about 35-ml menstrual blood containing cells and tissue fragments derived from the functionalis [4, 5]. Menstruation is followed by scar-free regeneration of the functional layer in the proliferative phase of the menstrual cycle, which is characterized by steadily increasing levels of the gonadotropins, follicle-stimulating hormone and luteinizing hormone, resulting in increased ovarian production and release of the steroid hormone estrogen [2]. Peak levels of these hormones trigger ovulation and formation of the corpus luteum, which produces progesterone. This steroid hormone dominates the endometrial changes during the second half of the menstrual cycle, the luteal phase. This phase, also called the secretory phase of the endometrium, is characterized by the transformation of endometrial glands into a secretory state, glandular glycogen storage and secretion, increased angiogenesis, vascular proliferation, and formation of spiral arteries [6]. While these changes serve to prepare the decidualized endometrium for implantation of the embryo in the case of fertilization, degeneration of the corpus luteum and the associated drop in progesterone and estrogen levels trigger menstruation if no fertilization occurs. This transition period between the late secretory phase and menstruation is accompanied by vasoconstriction and necrosis of the functionalis layer of the endometrium and by increases in prostaglandin and matrix metalloproteinase expression and activity, ultimately promoting controlled shedding of the functionalis layer [4, 7]. Intense research efforts in recent years have provided evidence for the concept that the highly regenerative nature of the endometrium is based on the activity of adult stem cells, as will be outlined in the following sections.

Current Evidence for Stem Cell Activity in the Endometrium

Stem Cells: A Brief Introduction

The human body is composed of approximately 210 different cell types [8]. This considerable diversity is initiated during embryonic development and is a consequence of complex differentiation processes. While differentiated cells, such as cardiomyocytes or pancreatic islet beta cells fulfill defined physiological functions within the organism,

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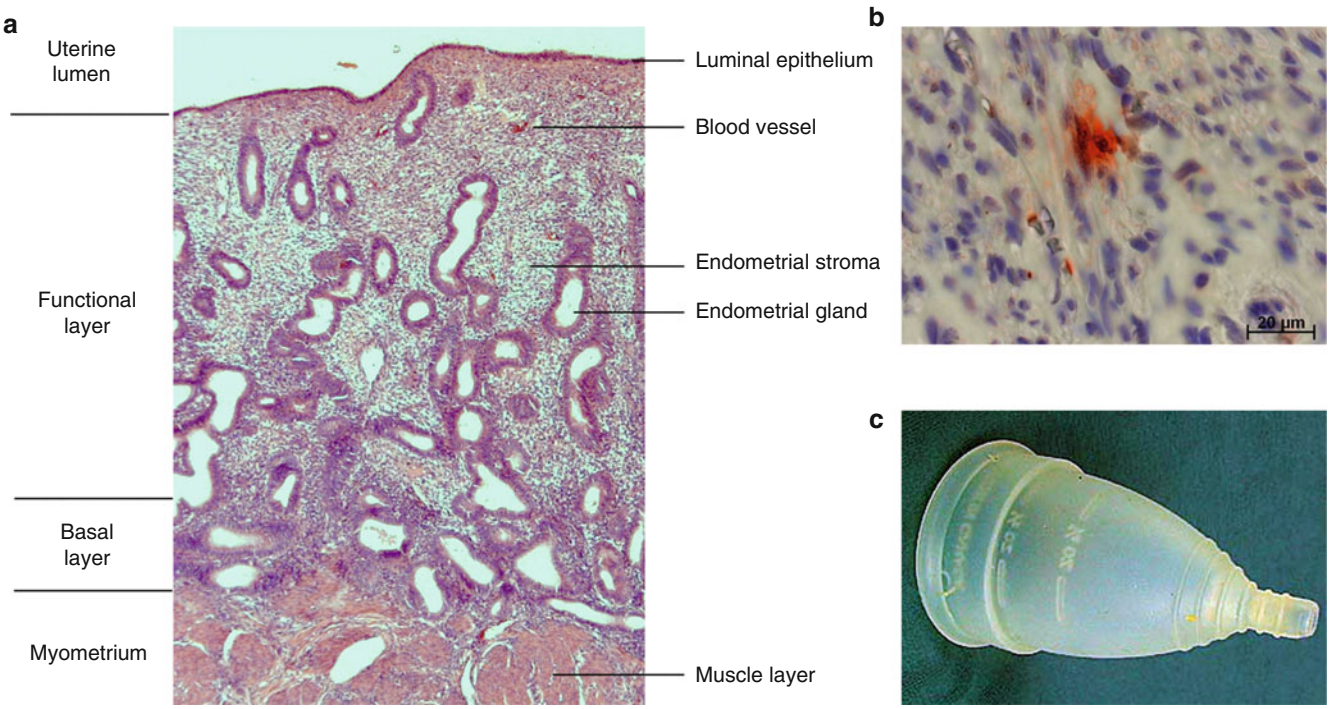

Fig. 7.1 Stem cells in the human endometrium. (**a**) Histological architecture of the human endometrium. Compared to the functional layer of the endometrium, which is shed during menstruation, the basal layer is characterized by a dense stroma. The muscular layer of the myometrium can be clearly distinguished from the endometrium. (**b**) Putative adult stem cells displaying positive immunostaining (*red*) for the RNA-binding protein Musashi-1 in the endometrial stroma. (**c**) The menstrual cup is a device frequently used to collect menstrual blood for MenSC isolation procedures

the only function of stem cells is the generation of precursors for these differentiated cell types. Thus, stem cells are undifferentiated cells that have the ability to self-renew and to generate more differentiated daughter cells through the process of asymmetric cell division (Fig. 7.2). Stem cells can be categorized based on their origin or on their differentiation potential.

Embryonic stem cells (ES cells) are derived from the blastocyst of the 3- to 5-day-old embryo and are pluripotent, being capable of differentiating into cells of all three germ layers (i.e., endoderm, ectoderm, mesoderm). In contrast, the zygote and its early cleavage stages up to the 16-cell stage can additionally form extraembryonic tissues and are thus called totipotent. Among the different types of stem cells, ES cells have the highest degree of developmental plasticity. They are clonally derived, maintain a normal karyotype in culture, are immortal, and can be propagated indefinitely in the embryonic state [9]. Regarding therapeutic applications, the property of ES cells to form teratomas and the ethically controversial issue of using cells derived from human embryonic tissue have limited their clinical use [10].

A cell type not associated with such strong ethical concerns, albeit with similar developmental plasticity is the induced pluripotent stem cell (iPS cell). In a landmark paper, Yamanaka and coworkers were able to convert human skin

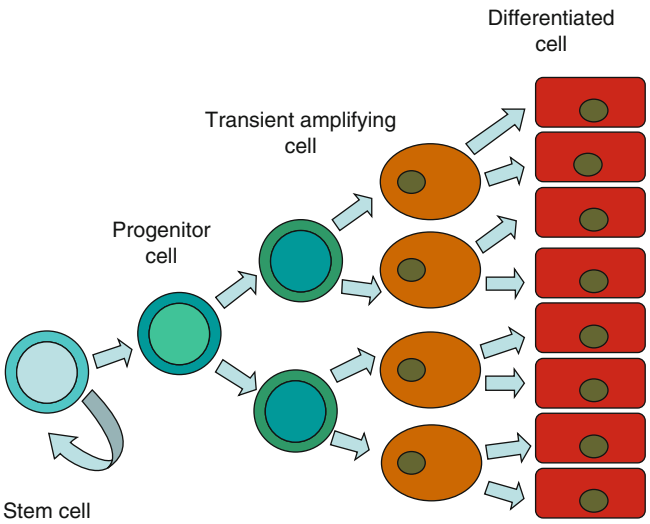

Fig. 7.2 Hierarchy of adult stem cells. The adult stem cell is multipotent and characterized by asymmetric cell division. The stem cell resides within a niche (not shown) which keeps the stem cell in an undifferentiated, slow cycling state. Cell division results in self-renewal and generation of a committed precursor cell, which is also called progenitor cell. Transient amplifying cells are progenitor cells characterized by high proliferative activity as a prerequisite for efficient tissue regeneration. Successive acquisition of differentiation markers and associated functional changes ultimately generate terminally differentiated cells which fulfill a specialized function within the human body

Table 7.1 Stemness-related marker expression of menstrual blood-derived stem cells

Marker	Reference
Oct-4	[16–19]
SSEA-4	[16, 17, 19, 20]
Nanog	[16, 17]
CD 9	[18, 21]
CD10 (CALLA)	[22]
CD13	[16, 20, 23]
CD29 (integrin β1)	[18, 20–23]
CD41a	[21]
CD44 (hyaluronan receptor)	[16, 18, 20–23]
CD49f (integrin α6)	[16]
CD54 (ICAM-1)	[23]
CD55 (decay accelerating factor)	[22, 23]
CD59	[21–23]
CD73 (ecto-5′-nucleotidase)	[16, 18, 20, 23]
CD90	[16, 20–22]
CD105 (endoglin)	[18, 20, 23]
CD117 (c-kit)	[16, 19, 24]
CD166 (ALCAM)	[16, 22]
TERT/telomerase	[16, 19, 21]

fibroblasts, i.e., a differentiated cell type, into a pluripotent state via transduction with the four transcription factors Oct3/4, Sox2, Klf4, and c-Myc [11]. Subsequent work showed that, depending on the cell type used as a source for iPS cell generation, less than four factors may be required to successfully generate these pluripotent cells [12]. Current research activities are focusing on methods of generating iPS cells without the necessity of retroviral transfections, which would be a major breakthrough regarding therapeutic applications in humans. Nevertheless, the property of tumor formation by pluripotent cells in vivo still represents a drawback of this technology [13].

In contrast to these pluripotent stem cells, adult stem cells show a more restricted developmental potential. They can either be multipotent, being capable of differentiating into multiple cell types of a given lineage, or unipotent, being only capable of generating one type of differentiated cell. Adult stem cells typically reside in an anatomical microenvironment called the stem cell niche [14]. Cells and extracellular matrix within the microenvironment provide signals to the stem cell which keep it in the undifferentiated state and which are able to trigger self-renewal and differentiation if necessary, e.g., when tissue regeneration is required during wound healing. This becomes apparent upon considering that some established cell surface markers of adult stem cells are actually matrix receptors, such as the hyaluronan receptor CD44 [15] or the β1-integrin CD29 (Table 7.1). Signals are mediated through different pathways, involving, e.g., β-integrins, TGF-β family members, the wnt-signaling pathway, or the notch pathway [14, 25, 26]. Upon asymmetric division of the stem cell, the daughter cells progressively undergo differentiation. These daughter cells are known as progenitor cells or committed stem cells. Transient (transit) amplifying cells are progenitor cells which – in contrast to the original stem cell – show a high proliferation rate. During repetitive cycles of cell division, these cells progressively acquire differentiation markers, ultimately resulting in acquisition of a terminally differentiated phenotype [27] which has a defined physiological function within the human body (Fig. 7.2). Although adult stem cells have a more limited developmental plasticity compared to embryonic stem cells, there are no major ethical concerns regarding the use of these cells for therapeutic purposes. The transplantation of bone marrow (as a source of hematopoietic stem cells) is a well-established example for a therapeutic application of adult stem cells that has become a routine procedure in clinical practice.

Endometrial Stem Cells: Early Concepts and Indirect Evidence

As pointed out earlier (section "The human endometrium during the menstrual cycle: physiology of a highly regenerative tissue"), the endometrium of reproductive-age women is a highly regenerative tissue, suggesting the presence of stem cell activity as an underlying principle. Early clinical observations of regeneration of functional endometrial tissue after complete endometrial ablation in monkeys and later in humans as well as metabolic radiolabeling studies in monkeys demonstrating the presence a germinal compartment localized to the lower basalis provided indirect evidence for the endometrial stem cell concept [28–30]. In addition, kinetic studies of endometrial cell proliferation have demonstrated zonal differences predicting an orderly replacement of differentiated endometrial glandular and stromal cells from slowly dividing putative stem cells residing in the basalis (reviewed in [1]). These findings were supported by analysis of changes in the methylation pattern of endometrial glands [31], the demonstration of clonality in endometrial glands based on PCR analysis of nonrandom X chromosome inactivation of the androgen receptor gene [32] as well as rare PTEN null mutations in individual endometrial glands [33]. At the functional level, the group of Caroline Gargett has performed pioneering work performing clonality assays on endometrial epithelial and stromal cells in vitro. In several key publications, her group could demonstrate that about 0.22 % of endometrial epithelial cells and about 1.25 of stromal cells are capable of forming individual colonies of >50 cells/colony within 15 days when purified single-cell suspensions of hysterectomy-derived endometrial tissue were seeded at clonal density [34]; define the growth factor requirements for culturing these cells under serum-free conditions [35], to characterize their surface marker expression profile (section "Marker expression profiles of endometrial

stem cells"); and demonstrate the in vitro differentiation potential of these cells [36]. Overall, these studies provide indirect yet convincing evidence for a stem cell activity in the human endometrium.

Marker Expression Profiles of Endometrial Stem Cells

Stem cells have frequently been phenotypically characterized based on the expression of a specific combination of marker genes which can be detected by flow cytometric analysis or PCR-based methods [37]. While specific cell surface receptor expression may reflect interaction of the stem cell with its niche, or the need for activation of particular signal transduction pathways, specific intracellularly located gene products associated with *stemness* are frequently transcription factors capable of steering complex cellular programs. In addition, surrogate markers of stem cell activity have been detected in endometrial stem cells. For example, the activity and expression of telomerase, associated with a suppression of replicative senescence, are higher in the endometrium compared to most other tissues in the human body [25, 38]. In addition, cells with the *side population* phenotype have been detected in the endometrium [39, 40]. Side population cells can be detected by flow cytometry based on the property of actively excluding fluorescent dyes such as Hoechst 33343 via the action of multidrug resistance proteins [37]. The side population is thought to be enriched in stem cells. About 2 % of the endometrium displays the side population phenotype [41]. Human endometrial side population cells and cell lines have been reported to display intermediate telomerase activity and express the markers Oct-4, GDF3, DNMT3B, nanog, and GABR3 (undifferentiated cells) [42]; CD105, CD146, CD90, WT1, cardiac actin, enolase, globin, and REN (mesenchymal) [40–42]; CD31, CD34, and KDR (endothelial) [40, 41]; and EMA (epithelial) [40]. The results of the study by Masuda et al. [41] suggest that different subpopulations such as cells predominantly expressing mesenchymal or endothelial progenitor cell markers exist in the endometrial side population. In addition, stromal clonal endometrial cells have been shown to express the markers CD146, CD140b (PDGFRβ), ITGB1 (CD29), CD44, NT5E (CD73), THY1 (CD90), ENG (CD105), W5C5, Msi1, Notch1, and SOX2 and to be negative for endothelial or hematopoietic markers such as CD31, CD34, and CD45 [36, 43, 44]. Finally, in human endometrial tissues, expression of the pluripotency markers Oct4, Sox2, nanog, and KLF4 [45, 46] and of components of the notch [25] (Fig. 7.1b) and wnt-signaling pathways [47] has been reported.

In Vitro and In Vivo Evidence for Endometrial Stem Cell Activity

The considerable differentiation potential and developmental plasticity of endometrial stem cells has been demonstrated in several independent in vitro studies. Clonal endometrial stromal cells were shown to be multipotent and to exhibit mesenchymal stem cell-like features, as they have been differentiated into smooth muscle cells, adipocytes, chondrocytes, and osteoblasts [36, 44, 48]. Furthermore, cells and cell lines derived from the endometrial side population could be differentiated into adipocytes and osteocytes [42, 49]. Endometrial side population cells could also be differentiated in vitro to produce gland (CD9+)- and stromal (CD13+)-like cells [39]. Further examples of a differentiation of endometrial stem cells into insulin-producing cells, cardiomyocytes, and dopaminergic neuron-like cell types will be presented in section "Therapeutic applications of tissue-derived endometrial stem cells".

The differentiation potential of endometrial stem cells is further underscored by their potential to generate endometrial tissue in vivo. For example, Cervello et al. [42] were able to generate endometrial-like tissue upon injection of 2×10^5–1×10^6 human endometrial side population cell lines into the kidney capsule of immunodeficient mice. While the data reported in the previous sections indicate that endometrial stem cells appear to have properties similar to typical tissue-resident mesenchymal [36, 44, 48], or possibly also endothelial [40, 41] progenitor cells, there is also functional evidence for a role of bone-marrow-derived progenitor cells in endometrial tissue regeneration: In a pioneering study, Taylor [50] reported about bone marrow transplantations between four human donors and recipients with an HLA mismatch and showed that donor-derived endometrial cells could be detected in endometrial biopsy samples from all bone marrow recipients, accounting for 0.2–48 % of epithelial cells and 0.3–52 % of stromal cells. Similar results were reported later for bone marrow transplants between male donors and female recipients, using the Y chromosome as a marker [51]. Although Cervello et al. [52] could recently confirm the presence of XY donor-derived cells in recipient endometrium in a different collective of bone-marrow-transplanted patients, their data suggested that bone-marrow-derived cells did not contribute to the endometrial side population of the recipient, leading the authors to the conclusion that XY donor-derived bone marrow cells may be considered a limited exogenous source of transdifferentiated endometrial cells rather than a cyclic source of donor-derived stem cells.

Menstrual Blood-Derived Stem Cells (MenSCs)

As the basal layer of the endometrium is not shed during menstruation, it has been postulated that it may be the preferential location of stem cells responsible for endometrial regeneration [29, 53]. While this concept is supported by the finding of zonal differences in endometrial cell proliferation (reviewed in [1]), and a differential glandular epithelial

expression of components of the wnt pathway in the basal versus functional layer of the endometrium [47], cells with adult stem cell-like properties have also been detected in and isolated from the superficial layers of the endometrium. For example, the adult stem cell marker Musashi-1 is expressed both in the basalis and functionalis layer of the endometrium, albeit with an enrichment of stem cells in the basalis [25]. Moreover, clonal endometrial stromal cells with mesenchymal stem cell-like marker expression profiles and multilineage differential potential have been isolated from the superficial layers of endometrial tissue obtained by routine biopsy techniques [44]. Similarly, endometrial side populations cells have been obtained by the Pipelle biopsy [42]. Moreover, the contribution of bone-marrow-derived cells to endometrial tissue regeneration [50, 54] additionally suggests that endometrial stem cell activity may not be exclusively localized to the basal layer. Finally, there are indications for a menstrual-cycle-dependent modulation of the functional state and release of adult stem cells: For example, an upregulation of circulating endothelial progenitor cells has been observed in a mouse model of endometriosis [55], a disease linked to altered endometrial stem cell function [25, 45, 54, 56]. Interestingly, the quantity and differentiation potential of these cells varies during the menstrual cycle in reproductive-age women: The number of CD133+/CD34− and CD133+/CD34+ progenitor cells and CD133+/CD34+/VEGF-R2+ endothelial progenitor cells per ml of blood was shown to fluctuate throughout the cycle in synchronization with circulating 17β-estradiol levels, and maturation of CD133+/VEGF-R2+ and CD133+/CD34−/VEGF-R2+ EPCs towards respective CD144+ advanced endothelial progenitor cell subpopulations was reduced at mid-luteal phase [57], demonstrating a relation between the release and functional properties of bone-marrow-derived progenitor cells and the menstrual cycle. Of note, the number of endothelial progenitor cell colony-forming units shows a negative correlation to the levels of stromal cell-derived factor-1 (SDF-1), a key cytokine for release and homing of bone-marrow-derived cells [58]. In this study, SDF-1 was also shown to be upregulated in the proliferative versus the secretory phase of the menstrual cycle. The concept of a menstrual-cycle-dependent release of endothelial progenitor cells is further supported by the finding that oral glucose-induced increase in circulating numbers of CD133+ and CD133+CD34+ cells and the endothelial differentiation potential of peripheral blood-derived endothelial progenitor cells is attenuated in insulin-resistant amenorrheic subjects [59]. Similarly, the number of Lin-/7AAD-/CD34+/CD133+/KDR+ circulating endothelial progenitor cells was found to be significantly higher in women with regular menstrual cycles compared to menopausal women and men [60]. Finally, a pilot study suggested a possible decrease in G-CSF-mobilized CD34+ hematopoietic stem cells during menstruation [61]. In light of these data, it is not surprising that endometrium-derived stem cells have been isolated from menstrual blood. The following sections will provide an overview over the isolation of MenSCs, their marker expression profiles and differentiation potential, and their therapeutic potential.

Isolation and Culture of MenSCs

Different protocols have been applied to collect and culture MenSCs. Although we use the general term MenSCs in this chapter, it has to be considered that these techniques may yield at least partially different stem cell populations, as the final enriched cell population may be derived either from different cell types of the endometrium (stromal vs. glands vs. endothelium vs. immune cells) or from the blood.

Meng et al. [21] collected 5 ml of menstrual blood in an antibiotic-containing solution using a urine cup-tubing method. They separated mononuclear cells by standard Ficoll centrifugation methodology and cultured these cells in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % FCS. While these cells were only marginally adherent after overnight culture, an outgrowth of adherent cells with a fibroblast-like morphology was observed after 2 weeks, with media changes performed twice weekly. For further studies, these so-called endometrial regenerative cells were clonally selected in 96-well plates. The basic protocol was later slightly modified to obtain MenSCs for a therapeutic feasibility study on human multiple sclerosis patients [62].

Hida et al. [22] collected mesenchymal cells from 10 ml of menstrual blood of six women (aged 20–30) on the first day of menstruation and cultured the samples in DMEM high glucose supplemented with 10 % FBS in two 10-cm dishes. A starting number of 1×10^7 cells were obtained, and the cells were subsequently subjected to adenoviral transfection for differentiation studies (see section "Myocardial infarction").

Phuc et al. [63] collected menstrual blood for 2–3 h using a menstrual cup (Fig. 7.1c). The blood was transferred into a Falcon tube containing PBS and fivefold antibiotic-antimycotic mix (Sigma) and kept on ice during transport. Samples negative for bacterial or mycotic contaminations were subjected to erythrocyte lysis and Ficoll-Paque purification of the mononuclear cell fraction, which was subjected to FACS-based cell sorting and in vitro differentiation into dendritic cells (see section "Generation of dendritic cells from menstrual blood cells").

A very detailed protocol for isolation of menstrual blood-derived stem cells has recently been published by Allickson et al. [16]. Menstrual blood was collected during the time of heaviest flow during the cycle using a menstrual cup, which was kept in place for less than 4 h. The blood was transferred to cooled sterile-buffered media containing heparin and antibiotics,

and cells were further processed in the presence of antibiotics (vancomycin, cefotaxime sodium, amikacin, gentamicin, and amphotericin B) within 24 h. For this purpose, cells were washed, concentrated, and ultimately cryopreserved by adding precooled 10 % dimethyl sulfoxide (DMSO), 30 % PBS, and 10 % human serum albumin to the cell suspension, aliquoted, and cryopreserved in a controlled rate freezer, prior to transferring them to a liquid nitrogen vapor storage freezer. For further applications, cells were thawed, cultured in Chang's complete media [64], and enriched for a subpopulation expressing the c-kit receptor CD117 using antibody-coupled magnetic beads (MACS sorting). In a study on 150 menstrual blood samples, the authors achieved an average yield of approximately 8×10^6 cells per preparation [16]. MenSCs displayed a fibroblast-like morphology.

Nikoo et al. [18] collected 3–5 mL of menstrual blood on day 2 of the menstrual phase from five women (22–45 years) using a menstrual cup. The samples were transferred to PBS containing amphotericin B, penicillin, streptomycin, and 2-mM EDTA, and mononuclear cells were separated using Ficoll-Paque. MenSCs were cultured in T75 flasks at 37 °C, 5 % CO_2 in DMEM-F12 medium containing the antibiotics mentioned above, and 20 % FBS. $1–1.5 \times 10^6$ mononuclear cells were recovered per milliliter of menstrual blood. The MenSCs had a stable fibroblast-like spindle-shaped morphology at later passages.

In summary, these studies demonstrate that MenSCs can be obtained using easy noninvasive procedures and that they can be cultured using routine cell biological techniques.

Evidence for MenSC Stemness

Adult stem cells are routinely characterized based on characteristic marker expression profiles, on their multilineage differentiation potential in vitro, and on additional properties such as long-term culturing properties and clonality. The defining criteria for multipotent mesenchymal stromal cells, for example, state that these cells must be plastic adherent when maintained in standard culture conditions; must express CD105, CD73, and CD90; and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules and that they must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [65]. As outlined in the following sections, MenSCs fulfill the criteria of a specific marker expression and of multilineage differentiation potential, in addition to showing long-term culturing properties in the absence of chromosomal aberrations.

Marker Expression Profiles

An overview of stem cell marker expression profiles of MenSCs is provided in Table 7.1. Meng et al. [21] demonstrated the expression of CD9, CD29, CD41a, CD44, CD59, CD73, CD90, and CD105 and the absence of monocyte and hematopoietic stem cell markers on mononuclear cell-derived "endometrial regenerative cells" isolated from menstrual blood. The cells did not express the embryonic stem cell markers SSEA-4 and nanog. In contrast, the CD117-positive MenSCs isolated by Allickson et al. [16] were characterized by the expression of the mesenchymal stem cell markers CD13, CD29, CD44, CD49f, CD73, CD90, CD105, CD166, and MHC class I and the pluripotent embryonic stem cell markers SSEA-4, nanog, and Oct-4. Furthermore, telomerase activity could be demonstrated comprising about 50 % of the activity of embryonic stem cells, similar to previous findings by Patel et al. [19], who also demonstrated expression of pluripotency markers, such as Oct-4, SSEA-4, and c-kit. The cells were negative for CD133, the endothelial progenitor marker CD34, and the leukocyte common antigen CD45. Zemel'ko et al. [20] reported isolation of MenSCs which were positive for expression of CD73, CD90, CD105, CD13, CD29, and CD44. Fifty percent of the cells were positive for the ES cell marker SSEA-4. MenSCs isolated by Nikoo et al. [18] were positive for the mesenchymal stem cell markers CD9, CD29, CD73, CD105, and CD44 and the ES cell marker Oct-4A, while CD34, CD133, CD45, CD38, and STRO-1 expression was not detectable. Menstrual blood-derived cells used for in vivo therapy of a mouse model of Duchenne muscle dystrophy displayed expression of CD13, CD29, CD44, CD54,CD55, CD59, CD73, CD90, and CD105 and were negative for CD133, CD34, CD45, and monocyte-macrophage antigens such as CD14, a marker for macrophage and dendritic cells [23]. Furthermore, the cells did not express CD31 (PECAM-1), CD50, or c-kit. The cell population was positive for HLA-ABC, but not for HLA-DR. Most recently, Khanmohammadi et al. [66] demonstrated that MenSCs show a marker expression profile which is very similar to bone-marrow-derived mesenchymal stem cells, including expression of CD44, CD73, CD90, and CD105. A notable exception was the high expression of the pluripotency factor Oct-4 in MenSC, which was not detected in the bone-marrow-derived cells.

While it has to be taken into account that differences in marker expression are at least partially due to physiological donor variability and the fact that the MenSC isolation procedures were not uniform, these studies demonstrate that MenSCs show a marker profile which is very similar to mesenchymal stem cells and that they frequently express the pluripotency factor Oct-4.

Functional Properties

Several independent studies have clearly demonstrated that MenSCs possess adult stem cell properties. The "endometrial regenerative cells" isolated by Meng et al. [21] could be maintained in tissue culture for >68 doublings without showing karyotypic abnormalities. Compared to umbilical cord-derived mesenchymal stem cells, these cells were

characterized by a higher proliferation rate and increased production of MMP3, MMP10, GM-CSF, angiopoietin-2, and PDGF-BB. The authors explored the differentiation potential of these cells by performing in vitro differentiation experiments. Although the authors reported successful differentiation into nine lineages (cardiomyocytic, respiratory epithelial, neurocytic, myocytic, endothelial, pancreatic, hepatic, adipocytic, and osteogenic), a potential caveat is associated with this study, as a proof for successful differentiation was only based on a single-marker immunostaining in many cases. Therefore, a more thorough investigation would have been worthwhile. Patel et al. [19] demonstrated that stromal cells derived from menstrual blood showed clonogenic properties and had the ability to differentiate into mesoderm- and ectoderm-derived tissues. The MenSCs isolated by Allickson et al. [16] could be subcultured up to 47 times before complete senescence and death. Karyotypic analysis demonstrated the maintenance of diploid cells without chromosomal abnormalities. Regarding the in vitro differentiation potential, the authors could clearly demonstrate the potential for multilineage differentiation with efficiencies ranging between 40 and 70 %: Adipogenic differentiation was confirmed by oil red O staining of lipid vacuoles, whereas osteogenic differentiation was demonstrated by alizarin red staining and qPCR for alkaline phosphatase expression. Chondrogenic differentiation was assessed by alcian blue staining for sulfated glycosaminoglycans, and neurogenic differentiation was demonstrated by neurofilament-3 and nestin PCR as well as positive immunocytochemistry for tubulin-III, glial fibrillar acidic protein, MAP-2, and nestin. In addition, cardiomyogenic differentiation was shown by immunocytochemistry of in vitro-differentiated cells for actin, desmin, troponin, and connexin 43. In accordance with these findings, a recent study indicated than MenSC have a chondrogenic differentiation potential which is very similar to bone-marrow-derived mesenchymal stem cells, albeit the mRNA expression patterns of the chondrogenic markers collagen 2A1, collagen 9A1, and SOX9 differed between the differentiated cells, depending on the tissue source of the stem cells [66].

Therapeutic Applications of MenSCs and Endometrial Stem Cells

In Vitro Differentiation Potential: MenSCs as a Therapeutic Cell Source

While the studies presented in section "Functional properties" have demonstrated the multilineage differentiation potential of MenSC, these differentiation experiments mainly served to provide proof of principle for the developmental plasticity of these cells. In contrast, more advanced studies have aimed at generating specific cell types for therapeutic purposes. In the following sections, we will present two of these approaches, the generation of cardiomyocyte-like cells and the generation of dendritic cells from MenSCs in vitro. Selected therapeutic applications of MenSCs and endometrial stem cells are listed in Table 7.2.

Table 7.2 Therapeutic application of MenSCs and endometrial stem cells

Disease	Stem cell source	Application	Reference
Myocardial infarction	MenSCs and immortalized endometrium-derived cells	Use of in vitro-differentiated cardiomyocytes in nude rat model of myocardial infarction	[22]
Diabetes (type 1)	Endometrial stromal-derived cells	Use of in vitro-differentiated insulin-producing cells in mouse model of diabetes	[67]
Diabetes (type 1)	Endometrial cells (hysterectomy)	Use of in vitro-differentiated insulin-producing cells in mouse model of diabetes	[68]
Diabetes (type 1)	MenSCs	Human phase 1/2 clinical trial announced	Clinicaltrials.org identifier NCT01496339
Parkinson's disease	Endometrial stromal cells (curettage)	Use of in vitro transdifferentiated cells in mouse model of Parkinson's disease	[69]
Multiple sclerosis	MenSCs	Safety study in four human MS patients by intravenous and intrathecal injection	[63]
Stroke	MenSCs	Use of in vitro in rat model of stroke	[17]
Duchenne muscular dystrophy	MenSCs	Use of myogenically in vitro-differentiated MenSCs in *mdx* mouse model	[23]
Critical limb ischemia	MenSCs	Use in mouse model of critical limb ischemia	[70]
Critical limb ischemia	MenSCs	Human phase 1/2 clinical trial announced	Clinicaltrials.org identifier NCT01558908
Liver cirrhosis	MenSCs	Actively recruiting human phase 1/2 clinical trial	Clinicaltrials.org identifier NCT01483248

Generation of Cardiomyocyte-Like Cells from MenSCs

Autologous cells with cardiomyocyte-like properties can be of considerable value for the treatment of myocardial infarction, one of the major causes of death in Western societies. A successful application of MenSC-derived cells in an animal model of myocardial infarction will be described in section "Myocardial infarction". By modifying the original protocol of Hida et al. [22], Ikegami et al. [71] were able to perform a cardiomyogenic differentiation of MenSCs under serum-free conditions. Murine fetal cardiomyocytes were initially cultured as feeders on laminin-coated plates in a medium consisting of M199 and medium containing 5-mM creatine, 2-mM L carnitine, 5-mM taurine, 0.1-nM thyroxin (T3), 0.1-mM insulin, 2.5-mM pyruvate, and 2-mg/mL heat-treated bovine serum albumin (Fraction V). MenSCs were subsequently cultured at a density of 3×10^3 cells/cm^2 for cardiomyogenic induction. The previous study by Hida et al. [22] had indicated that no 5-azacytidine was necessary to promote differentiation. Cells started beating after 3 days and were immunopositive for cardiac troponin-I, α-actinin, and connexin 43 at evaluation after 1 week of induction. Successful cardiomyogenic differentiation was demonstrated at the functional level by analysis of fractional shortening of the cell and of action potentials, which were shown to be more physiological using serum-free differentiation compared to serum-containing media. Apart from the increased functionality of the differentiated cells, the new protocol presents a major advance for potential therapeutic applications, as it eliminates safety issues linked to the use of animal-based sera.

Generation of Dendritic Cells from Menstrual Blood Cells

Based on their immunomodulatory properties, dendritic cells are currently being used in the immunotherapy of cancer and for the purpose of cancer vaccination [72, 73]. The therapeutic approach involves the in vitro generation of dendritic cells from a patient's monocytes or hematopoietic stem cells, and a reintroduction of these autologous cells into the patient after stimulation with tumor-specific antigens. While previous studies have relied on the use of peripheral blood- or umbilical cord blood-derived monocytes or hematopoietic stem cells from bone marrow and blood sources, recent work by Phuc et al. [63] has shown that these cells can also be generated from menstrual blood-derived cells. The authors used the mononuclear cell fraction of menstrual blood as a source for a FACS-based sorting of CD4+ T cells and monocytes. Employing a two-step in vitro differentiation protocol involving culturing of menstrual blood-derived monocytes in the presence of IL-4 + GM-CSF to generate imDCs, and a subsequent 24-h exposure to TNF-α, the authors could generate dendritic cells characterized by their characteristic morphology; expression of HLA-DR, HLA-ABC, CD80, and CD86; IL-12 release; uptake of dextran-FITC and by their capability to stimulate allogeneic T-cell proliferation. Although the authors emphasized the advantage of using a quantitatively attractive noninvasive source for dendritic cell generation, the yield after differentiation was still comparably low (68 %). Therefore, it remains to be shown if menstrual blood-derived dendritic cells will be a viable alternative for more traditional established cell sources.

Therapeutic Efficacy of MenSCs in Animal Models of Disease

In the following sections, examples for a successful therapeutic application of human MenSCs in established animal models of human diseases will be presented. Two major conceptual differences become apparent in these studies: One frequently used approach involves a pre-differentiation of MenSCs into a defined cell type which is used to replace the respective damaged cell type in the disease model. In light of envisaged therapeutic approaches in humans, this approach may be the more reliable and safer one, provided that the transplanted cells retain phenotypic stability in the new microenvironment. The second approach involves the direct application of undifferentiated MenSCs in a particular disease model. While this therapeutic route may lead to less predictable and more indirect effects, it has nevertheless proven to be equally efficient at least in some disease models.

Myocardial Infarction

Heart disease is the leading cause of death in the USA and constitutes a major epidemiological challenge [74]. Therefore, the application of novel therapeutic approaches involving stem cells has gained considerable attention in recent years. A pioneering study on the therapeutic use of MenSCs in an animal model of myocardial infarction was published in 2008 by Hida et al. The researchers isolated MenSCs (section "Isolation and culture of MenSCs"), transfected them with an enhanced green fluorescent protein (EGFP) adenoviral construct (for detection purposes only), and achieved cardiocytic differentiation using a coculture system with embryonic cardiomyocytes (see section "Generation of cardiomyocyte-like cells from MenSCs"). Transdifferentiation was not due to fusion-dependent cardiomyogenesis, as demonstrated by chromosome analysis. In vitro, about 50 % of the differentiated MenSCs started beating strongly, spontaneously, and synchronously. By immunostaining, expression of the marker protein troponin-I, a striated staining pattern for α-actinin, and a diffuse dot-like staining pattern of connexin 43 around the margin of each cardiomyocyte were demonstrated, suggesting tight electrical coupling. Analysis of action potentials revealed cardiomyocyte-specific sustained plateaus and slowly depolarizing resting membrane

potentials, i.e., cardiomyocyte-specific pacemaker potentials. The authors subsequently employed the transdifferentiated MenSCs in a nude rat model of myocardial infarction based on ligation of the left anterior coronary artery. In the therapeutic group, $1–2 \times 10^6$ EGFP-positive MenSCs were injected into the center and margin of the infarcted myocardium. The area of the myocardial infarction was significantly lower in the MenSC-treated group compared to controls injected with bone-marrow-derived mesenchymal stem cells. Of note, the EGFP-positive MenSCs in the myocardial infarction area displayed a clear striation staining pattern of cardiac troponin-I and sarcomeric α-actinin, suggesting a high degree of in situ cardiomyogenic transdifferentiation as an underlying cause of improved cardiac function. While nuclear fusion between the cocultivated MenSCs and murine cardiomyocytes without separation of the athelocollagen membrane was observed in only 0.16 %, the potential contribution to cell fusion in the in vivo model is not fully clear at this point. Overall, the data of this study open up exciting therapeutic perspectives, as the authors suggested that MenSCs could be obtained in a noninvasive manner from young female volunteers and stored to obtain a MenSC bank covering all HLA types for cardiac stem cell-based therapy. For applications in humans, the development of serum-free differentiation protocols represents an important advancement (section "Generation of cardiomyocyte-like cells from MenSCs"); however, carefully controlled protocols will be required as a coculture with animal-derived cells is still part of the protocol [71]. Nevertheless, this carefully executed study provides an exciting starting point for additional investigations in preclinical models.

Stroke

Stroke is the fourth leading cause of death in the USA [74]. Within the infarcted brain region, tissue in the ischemic core is mostly irreversibly damaged, while the tissue in the surrounding penumbra area may be rescued provided that proper therapeutic action is taken in a timely manner [75]. As the major current pharmacological therapy for stroke involves treatment with tissue plasminogen activator within a very narrow time window of 3 h, the alternative therapeutic application of stem cells for stroke is an area of intense research. Stem cells may exert a beneficial effect during the later inflammatory phases of the disease when tissue repair commences and may promote angiogenesis and survival of neurons via secretion of neuroprotective factors [76]. In fact, apart from the use of ES cells, beneficial effects of adult stem cell therapy in experimental models of stroke have been reported for bone marrow [77], umbilical cord blood [78], adipose tissue [79], and stem cells isolated from amniotic fluid [80]. In 2010, Borlongan et al. could demonstrate the therapeutic efficacy of MenSCs in an in vivo model of stroke [17]. Using a modification of the procedure initially described by Patel et al. [19] (section "Isolation and culture of MenSCs"), the authors collected on average 8–10 ml of menstrual blood at the time of the heaviest flow (day 1–3) prior to cryopreservation. Cells were later expanded and immunoenriched for CD117 expression to preselect for a highly proliferative and vital subpopulation of MenSCs. Cultures of MenSCs expressed the ES cell markers Oct-4, SSEA-4, and nanog, as detected by immunocytochemistry, and the chemokine receptor CXCR4, which was also expressed on ES cells. Using a differentiation protocol involving culture in neural induction medium (DMEM/F12 containing N2 and FGF-2) and a subsequent stimulation with retinoic acids, the authors could induce expression of the neural markers MAP2 and nestin in the MenSCs. A large proportion of cells acquired an astrocytic phenotype (GFAP positivity) upon FGF-2 withdrawal. Coculture of primary rat neurons with MenSCs or conditioned media derived from these cells significantly protected the neural cells from oxygen-glucose deprivation (OGD), an in vitro model of stroke. ELISA analysis of conditioned media of OGD-exposed cultured MenSCs revealed an upregulation of several trophic factors, including vascular endothelial growth factor, brain-derived neurotrophic factor, and neurotrophin-3. As these factors have previously shown beneficial effects in stroke [81, 82], their upregulation may be mechanistically linked to the neuroprotective effect of the MenSCs in the in vitro model. Notably, a protective effect was also observed in an established rat model of stroke based on the induction of transient unilateral focal ischemia as achieved by middle cerebral artery occlusion [83]. MenSCs were applied either intercerebrally or intravenously 2 h after the ischemic insult, exerting a neuroprotective effect and leading to a significant improvement in motor asymmetry, motor coordination, and neurologic tests. Immunohistochemistry employing human-specific antibodies revealed survival of the transplanted cells in the penumbra 14 days after transplantation. However, as over 90 % of these MenSCs still expressed stem cell markers such as Oct-4, secretion of neuroprotective factors rather than replacement of damaged tissue appeared to be the mechanistic principle behind the beneficial effect of MenSC application in this experimental stroke model. Thus, although these results are very encouraging, more experimental data are needed to fully understand the pathophysiological role of MenSC in experimental stroke therapy.

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked inherited form of muscular dystrophy caused by mutations or exon deletions in the dystrophin gene, which encodes a protein conferring structural stability to the dystrophin complex. This multiprotein complex links the intracellular cytoskeleton of muscle cells to the extracellular matrix, thus providing physical strength and mechanical support for the contractile forces

generated by the muscle fiber [84]. Affecting approximately one in 3,500 newborn males, the disease is characterized by progressive muscle weakness, ultimately resulting in paralysis and an average life expectancy of only 25 years [85]. As the underlying molecular cause of the disease is known, intense research efforts are undertaken to cure the disease and have yielded promising results in experimental and preclinical models. Apart from pharmacological approaches, classical gene replacement therapy, and antisense-mediated exon skipping, stem cells have been applied for therapeutic purposes [85]. In a pioneering paper published in 2007, Cui et al. demonstrated a therapeutic effect of menstrual blood-derived cells in a well-established mouse model of DMD [23]. *mdx* mice lack dystrophin in skeletal muscle fibers; however, the phenotype of the mice is more mild compared to human DMD [84]. In cell culture, menstrual blood-derived cells selected for therapeutic purposes showed a stromal cell-like morphology and expressed a mesenchymal cell surface marker spectrum similar to MenSC preparations reported by other laboratories [23] (section "Marker expression profiles"). Successful 5-azacytidine-induced myogenic differentiation could be achieved in vitro, based on the expression of the markers MyoD, desmin, myogenin, and dystrophin. When untreated menstrual blood-derived cells were implanted directly into the thigh muscles of *mdx*-scid mice, myotubes expressing human dystrophin as a cluster could be detected after 3 weeks. Immunohistochemistry with an antibody against human nuclei and DAPI revealed that dystrophin-positive myocytes had nuclei derived from both human and murine cells, suggesting that dystrophin expression could be attributed to fusion between murine host myocytes and human donor cells, rather than myogenic differentiation. This view was supported by further in vitro studies demonstrating fusion between human endometrial cells and cocultured murine C2C12 myoblasts.

Critical Limb Ischemia

Critical limb ischemia, an advanced form of peripheral artery disease, comprises medical conditions such as chronic ischemic rest pain, ulcers, or gangrene caused by proven occlusive disease [86, 87]. As up to 45 % of the affected patients require amputation, there is considerable interest in the development of therapies efficiently reestablishing blood flow in the affected limbs. Besides surgical intervention and application of proangiogenic cytokines, therapeutic application of bone-marrow-derived mesenchymal stem cells and endothelial progenitor cells has yielded promising results in vivo [87, 88]. Application of the stem cells resulted in increases in angiogenic factor secretion and increased angiogenesis both in animal models and in several small-sized clinical studies [89, 90], demonstrating its benefit as a supportive therapy especially for patients without therapeutic options. In 2008, Murphy et al. [70] expanded this therapeutic repertoire by applying MenSC in a mouse model of critical limb ischemia: Using "endometrial regenerative cells" isolated according to the protocol developed by Meng et al. [21] (section "Isolation and culture of MenSCs"), the authors first demonstrated a substantial proliferative effect of these MenSC conditioned media on human umbilical vein endothelial cells, which was attributed to the secretion of proangiogenic factors such as PDGF-BB and angiopoietin. In a pilot study on 16 mice, 1×10^6 MenSC were injected intramuscularly after ligation of the femoral artery and its branches for induction of limb ischemia. To additionally reproduce a neurotrophic ulcer-like injury, the *N. peroneus* was excised in the experimental animals. Injections of MenSC in the treatment group were repeated on day 0, day 2, and day 4. As a result of the experimentally induced ischemia, necrosis was observed in the legs of the control mice after 14 days, whereas all MenSC-treated mice had intact limbs, albeit two mice displaying signs of impeded walking. The results of this pilot study suggest that MenSC may be a viable alternative to bone marrow stem cells in critical limb ischemia. As the authors have registered a clinical trial on a small patient collective (see section "Therapeutic efficacy of MenSCs in animal models of disease"), data on the therapeutic efficacy of these cells should be available in the not too distant future.

Alterative Endometrial Stem Cell Sources

The previous chapters have demonstrated that stem cells derived from menstrual blood are a readily available source of adult stem cells which can be obtained by noninvasive techniques. However, alternative sources of endometrial stem cells have been utilized to gain additional knowledge on the function and therapeutic potential of these cells. Initial studies relied on hysterectomy-derived endometrium [1], which had the advantage of containing a high amount of endometrial stem cells, but also the disadvantage of limiting the number of patients available for clinical correlation studies on endometrial stem cells. Subsequent studies demonstrated that endometrial stem cells could also be obtained by minimally invasive superficial endometrial biopsies, which may constitute an alternative to MenSCs, as this technique allows for obtaining endometrial stem cells independent of the menstrual cycle [42, 44].

Another highly interesting feature of endometrial stem cells is the finding that they are more amenable to reprogramming into induced pluripotent stem (iPS) cells compared to skin fibroblasts [91], which can be attributed to the high basal expression levels of pluripotency markers [44, 45]. Of note, Nishino et al. [92] were able to successfully generate iPS cells from MenSCs. As the issue of teratoma formation of iPS cells still constitutes a major obstacle for therapeutic applications [93], we will focus on the therapeutic application of multipotent non-menstrual blood-derived endometrial stem cells in the following section.

Therapeutic Applications of Tissue-Derived Endometrial Stem Cells

Apart from MenSCs (section "Therapeutic efficacy of MenSCs in animal models of disease"), tissue-derived endometrial stem cells obtained by low invasive biopsy or hysterectomy have been employed for therapeutic purposes in several animal models of disease. For example, the study by Hida et al. [22] not only demonstrated therapeutic efficacy of MenSCs in a mouse model of myocardial infarction but also showed a similar therapeutic effect upon application of in vitro-differentiated endometrial cells derived from hysterectomy tissue (section "Myocardial infarction"). In contrast to MenSCs, the tissue-derived cells required retroviral transfection with HPV16E6, E7, and hTERT, which constitutes a problem regarding therapeutic applications in humans.

Wolff et al. [69] described the successful therapeutic application of endometrial stem cells in an animal model of Parkinson's disease. This common neurodegenerative disease is caused by a progressive loss of dopamine-producing neurons in the substantia nigra projecting into the striatum, which ultimately results in tremor, hypokinesia, and rigidity in affected patients [94, 95]. Wolff et al. [69] initially isolated endometrial stromal cells obtained by curettage from nine reproductive-aged women. After repeated passaging, the cells were strongly positive for CD146, CD90, and PDGFRß1 and negative for CD31 and CD45. Using an in vitro differentiation protocol involving sequential culturing in the presence of FGF and EGF and subsequently butylated hydroxyanisole, dibutyryl cyclic AMP, 3-isobutyl-1-methyl-xanthine, and all-trans-retinoic acid [96], the authors were able to obtain dopaminergic neurons. The cells exhibited axon projections, pyramidal cell bodies, and dendritic projections; expressed the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase, and the neural marker nestin; and displayed electrophysiologic properties specific to dopamine-producing neurons. These cells were transplanted into the striatum of both immunocompetent and immunodeficient mouse models of Parkinson's disease based on the injection of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine. Successful transplantation was confirmed by detection of human-specific DNA, mitochondrial markers, and human nestin. The endometrium-derived cells were able to migrate into the brain lesion, populating both the striatum and the substantia nigra. Notably, the researchers could show that the endometrium-derived cells had transformed into dopamine-producing neurons, resulting in partially restored dopamine levels in vivo. While the results of this study demonstrate the plasticity and therapeutic potential of endometrial stem cells, future studies still need to address if the increased dopamine levels suffice to improve the clinical symptoms, in particular the coordination of balance and movement in this mouse model.

Two independent studies have recently explored the therapeutic potential of endometrial stem cells in experimental models of type I diabetes, a disease characterized by insulin deficiency caused by a loss of pancreatic ß-cell function [97]. Li et al. [68] used an immunodepletion protocol to isolate mesenchymal stem cell-like cells from hysterectomy tissue of 11 patients. The cells were positive for Oct-4, nanog, nestin, CD44, CD29, CD105, and CD81 and showed multilineage differentiation potential in vitro. After a 7-day culture period in pancreatic differentiation medium, the authors observed the formation of cell clusters and aggregates, which exhibited a pancreatic cell-like gene signature. Quantitative real-time PCR analysis confirmed transcriptional upregulation of typical pancreatic cell markers, including insulin, Glut2, Pax4, Nkx2.2, NeuroD, Isl-1, somatostatin, and glucagon, compared to undifferentiated cells and endometrial fibroblasts. Notably, insulin and c-peptide secretion in response to a glucose stimulus were significantly increased in the differentiated cells. The therapeutic potential of the endometrium-derived cells was further evaluated in streptozotocin-induced hyperglycemic mice, an established model of diabetes mellitus. Following injection of 2×10^7 differentiated endometrial stem cells into the subcapsule of the left kidney, the formation of diffuse aggregated islet-like clusters which were immunopositive for insulin and glucagon was observed. Human insulin levels were readily detectable and stable during an 8-week observation period, whereas blood glucose levels were significantly decreased in the transplanted mice compared to controls. Of note, no expression of c-Myc or Oct-4 was detected in the islet-like tissues, and no teratoma formation was seen after 12 weeks of observation. A second study by Santamaria et al. [67] employed a similar approach. The authors differentiated human endometrial stromal stem cells in vitro and succeeded in generating cells which expressed the markers PAX4, PDX1, GLUT2, and insulin. Insulin was secreted in a glucose-dependent manner. In a diabetic mouse model, transplantation of the differentiated endometrial cells resulted in an increase in human insulin levels, which was accompanied by a stabilization of blood glucose levels within 5 weeks. Notably, mice transplanted with undifferentiated cells developed progressive hyperglycemia, whereas control mice lost weight and developed cataracts as a result of insulin deficiency and hyperglycemia. In summary, the promising results of these animal studies suggest that the therapeutic application of in vitro-differentiated endometrial stem cells may be a future alternative to pancreatic islet transplantation, which is currently limited by availability of cadaveric donor tissue [98].

Clinical Perspective

As the previous sections have demonstrated, the application of MenSCs and endometrial stem cells in in vitro and animal models of human diseases has yielded highly

promising results. In contrast to the application of ES or iPS cells, the risk of inadvertently causing a malignant disease as a side effect of the stem cell therapy appears to be low [21]. Moreover, for some female patients, autologous stem cell transplantations are feasible, avoiding possible immunological incompatibility and rejection issues. However, in order to make therapeutic options available for postmenopausal women and men, a systematic immunophenotyping and banking of MenSCs would be required. In addition, the symptoms of most diseases potentially amenable to stem cell therapy will become apparent only at a postmenopausal age, establishing a requirement for MenSC banking even in the case of autologous therapies. In urgent cases, endometrial stem cells obtained by superficial endometrial biopsy [44] could serve as an alternative source for autologous cell transplantations in women. Furthermore, in vitro expansion of the MenSCs will very likely be required in order to obtain a sufficient amount of cells for transplantation. In spite of the proliferative character of MenSCs, sufficient cell numbers may not be easily achieved using menstrual blood derived from only one donor. The development of serum-free culture conditions for MenSCs is therefore an important step towards their use in clinical applications [71]. The field of MenSC therapeutics can benefit from decades of experience regarding GMP procedures, surface antigen characterization, cell banking, and standardized procedures developed in the fields of transfusion medicine and transplantation immunology. Borlongan et al. [17] pointed out that MenSCs express a similar marker spectrum as mesenchymal stem cells known for their immunosuppressive effects. They furthermore reported that MenSCs showed a very weak stimulatory response in a mixed lymphocyte reaction. In line with these findings, a systematic study by Nikoo et al. [18] demonstrated that MenSCs affected the proliferative response of peripheral blood mononuclear cells in allogeneic mixed lymphocyte reaction in a dose-dependent manner. The results of their study prompted the authors to suggest that MenSCs may even be employed as a therapeutic option to prevent or modulate graft-versus-host disease in allogeneic transplantation. Nevertheless, the specific requirements for clinical applications within this new field of regenerative medicine need to be carefully defined and need to result in unified procedures at least at the national level. Furthermore, access to MenSC banks in order to locate an appropriate donor will have to be standardized similar to existing systems in the field of transplantation immunology. Apart from the issue of immunocompatibility, other safety precautions need to be taken. Similar to the experimental studies discussed in section "In vitro differentiation potential – MenSCs as a therapeutic cell source", it has to be ensured that the MenSCs maintain their phenotypic stability prior to therapeutic use in humans. For example, cells containing chromosomal aberrations should obviously not be used for therapeutic purposes, requiring defined regular screening procedures. Moreover, the risk of viral and especially microbial contaminations can be expected to be higher in MenSCs compared to blood- or bone-marrow-derived adult stem cell sources, requiring an equally important strict and efficient control system. Allickson et al. [16] have provided a good example for a thoughtful MenSC isolation and storage protocol which addresses several of the issues mentioned above. In addition, detailed descriptions of endometrial stem cell and MenSC characterization and isolation protocols have been provided in several patent applications, including US patent applications #20090053182 (MenSC/endometrial stem cell isolation and characterization), #20080241113 and #20100040588 (isolation and cryopreservation of MenSCs), as well as #20110268710 (treatment of stroke with MenSCs). Finally, Zhong et al. [62] defined the release criteria of MenSCs to be used in allogeneic transplantations in humans: (i) the absence o bacterial and mycoplasma contamination; (ii) endotoxin levels <1.65 EU/ml; (iii) morphology consistent with adherent, fibroblastic-like shape; (iv) CD90 and CD105 positive (>90 %) and CD45 and CD34 negative (<5 %) by flow cytometry; (v) cell viability >95 % by trypan blue staining and flow cytometer; (vi) and the absence of karyotypic abnormalities.

Keeping these caveats in mind, a number of pilot studies and clinical trials on MenSC safety and therapeutic efficacy in humans have already been initiated. A pioneering pilot study explored the feasibility of MenSC transplantation in four patients suffering from multiple sclerosis [62]. Donors were selected after rigorous testing according to federal regulation 21 CFR1271 of the US Federal Drug Administration, and MenSCs were isolated essentially as described by Meng et al. [21] (section "Isolation and culture of MenSCs"). In vitro expansion of the MenSCs allowed for obtaining 100–200-million cells after three to four passages. Cells that passed the release criteria (see above) were injected either intravenously or intrathecally in clinical grade saline containing 50 % autologous heat-inactivated serum. The four multiple sclerosis patients participating in the trial received between 16- and 30-million MenSCs applied in several discrete doses of either three million or six million cells. After a follow-up of 2–12 months and monitoring by physical exam, CBC/biochem panel, fecal occult blood testing, chest X-ray, and tests for PSA, CEA, and alpha fetoprotein, none of the patients described notable events or abnormalities, with the exception of a mild self-limiting headache in one patient, which was ascribed to the lumbar puncture procedure. Moreover, during the observation period, no disease progression was noted. While the study is limited by the small patient number, it nevertheless shows that MenSC transplantation appears to be safe if the cells are well characterized and appropriate precautions are taken.

The NIHs public registry and results database ClinicalTrials.gov lists several clinical trials related to the use of menstrual blood-derived or endometrial stem cells. While some of these

trials have a research focus, aiming at obtaining new information on the properties of these cells, others are directed towards direct therapeutic applications. For example, a prospective observational study headed by Erin Wolff of the National Institutes of Health Clinical Center in Bethesda, USA, recruits normal controls, patients with rare diseases or reproductive disorders, and patients who have undergone hematopoietic stem cell transplant (planned enrollment: $n=100$) to investigate endometrial biopsies with the aim of a better understanding of bone marrow cell engraftment of the uterus (ClinicalTrials.gov identifier NCT01468935). A prospective observational study initiated by Carlos Simón of the IVI Valencia, Spain, recruits endometriosis patients ($n=30$) with the purpose of characterizing endometrial stem cells through flow cytometry and side population techniques and in vitro cellular proliferation assays to learn more about the involvement of endometrial stem cells in this disease, which is associated with reduced fertility and pain symptoms (identifier NCT01412138). As stem cell marker expression is dysregulated in endometriosis [25, 45], this study follows a good rationale. Among the therapy-oriented studies, an interventional study announced by Medistem Inc. aims at assessing the safety and feasibility of using endometrial regenerative cells/MenSCs in 15 patients with critical limb ischemia that are not eligible for surgical- or catheter-based interventions (identifier NCT01558908). In this phase 1/2 trial, patients are planned to be treated with increasing doses of 25-million, 50-million, or 100-million MenSCs by intramuscular injection. MenSC treatment will be followed by assessment of treatment safety and improvement of clinical symptoms including ulcer healing, rest pain, and reduction in amputation. Moreover, the potential of MenSCs for the treatment of type 1 diabetes will be addressed in an interventional study headed by Charlie Xiang, First Affiliated Hospital of Zhejiang University, China, and S-Evans Biosciences Co. Ltd (identifier NCT01496339). Both safety of MenSC application and improvement of diabetes mellitus-associated parameters (HbA1c, hypoglycemic events, C-peptide, blood glucose levels, etc.) will be investigated in this phase 1/2 trial. Finally, an actively recruiting interventional phase 1/2 study initiated by S-Evans Biosciences Co. Ltd aims at testing the efficacy and safety in patients with liver cirrhosis by comparing conventional therapy plus MenSC transplantation with conventional treatment in a placebo group (identifier NCT01483248). Taken together, the interventional studies can be expected to provide valuable information on the safety and therapeutic efficacy of MenSC transplantation in a clinical setting in the near future.

Summary and Outlook

Menstrual blood contains a pool of multipotent mesenchymal stem cell-like cells which are characterized by the additional expression of pluripotency factors such as Oct-4. These highly proliferative cells can be obtained by simple noninvasive procedures and can be successfully expanded in vitro for prolonged periods without acquiring chromosomal aberrations. In contrast to ES cells, no ethical controversy is associated with their use in therapeutic applications. Furthermore, animal studies suggest that no increased risk of tumor formation is associated with their in vivo use, in contrast to pluripotent ES and iPS cells, which have the inherent risk of forming teratomas in vivo. MenSCs show a low immunogenicity, are suitable for allogeneic transplantation, and may even have a positive influence on the immune system of the recipient. MenSCs show multilineage differentiation potential in vitro and in vivo and have been successfully employed in several animal models of human disease. Preliminary data from clinical pilot studies have demonstrated that their in vivo use in human recipients is safe. Ongoing phase 1/2 clinical trials on the therapeutic efficacy and safety on MenSC applications in human patients raise the hope that the potential of these attractive adult stem cells can be fully assessed in the not too distant future. While MenSCs harbor the potential for autologous transplantation at least for reproductive-age female patients, the majority of potential patients would greatly benefit from MenSC cell banking. Consequently, there is a need for defining unified preparation procedures and safety requirements regarding the clinical use of these cells and for providing an informational infrastructure that allows to locate matching donor cells for a specific patient in need of MenSC therapy. As similar procedures and infrastructures are already well established in other areas of stem cell therapy, a successful translation of the exciting recent research findings on MenSCs and endometrial stem cells into clinics should be feasible within a short timeframe.

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Part III

Fat and Stem Cells

Use of Animal Fats in Traditional Chinese Medicine

8

Ping Chung Leung

Introduction

I believe our editor's plan is to organize the most comprehensive program for the discussion on stem cells and tissues related to stem cells. Since mesenchymal stem cells from the bone marrow differentiate into important components of blood, cells related to bones, and fat itself and many of these cells are actively cross convertible, a conceptual assumption that even well-formed animal fat could be having stem cell function might stand. Henceforth, when animal fats are used in traditional medicine, the indication could be related to our modern concept of tissue regeneration through an unrecognized utilization of the innate stem cell properties. I intend to explore the possibility of this assumption.

Searching through the classics of Traditional Chinese Medicine, one does find great varieties of animal fats being used for divergent therapeutic purposes, some of which are even related to tissue regeneration. This practical use is most likely coincidental rather than being attributable to "ancient wisdom." One has to realize that in the primitive agrarian society of China, animal food is a rarity available only to the rich people. Some peasants could enjoy meat in their meals only a few times per year, only at important festive cerebrations. While modern communities of today consider animal fats harmful to health, thus discarding them, peasants in ancient China would value all the components of animal meat and would never consider sacrificing fats as a waste. Indeed, on the contrary, for those who seldom get access to meat in their normal diet, the fat within the animal meat could be a delicacy. Some delicate dishes in the Chinese Gomez Recipe have chosen lumps of pure animal fats as the major component!

Actually, for the ancient healers in China, one important principle exists: they believe that food and therapeutic agents coexist. In other words, nutritional items are considered also having therapeutic values. When animal fats are consumed through the alimentary tract, not only is gastronomical value expected, but some practical health promotion effects are desired. While little was known about the digestive and assimilation effects of the exocrine secretions in the gastrointestinal tract, ancient healers tend to identify consumed items as being capable of supplementing what was being deficient. Thus, animal fats were recommended to supplement deficiencies. Since the oral route was the only common route of therapeutic administration, the ingested food, or therapeutic agent, after going through the complicated procedures of digestion, would have been disintegrated to different degrees and could hardly cast any influence comparable to our current expectation for stem cells. Animal Fats in Chinese Medicine, therefore, should in no way be related to stem cells.

When animal fats are used by traditional healers externally, particularly when there are breakages of epithelial surface and direct contacts between the fat and body tissues occur, it would be easier to make assumption that some degrees of molecular interaction might be possible. Under such circumstances, it is yet necessary to ignore the fundamental differences in the genomic pattern of different animals.

Actually, mesenchymal stem cells do have different channels of differentiation, into either hemopoietic, bone-related, or fat cells. The fat cells in the marrow are not similar to those outside the marrow in the periphery. Now that marrow fat cells have been identified as being involved in endocrine-like function, the difference between them and peripheral fat has become even more obvious. While Traditional Chinese Medicine practice utilizes animal fat for clinical problems, the justifications in no way will be related to present-day scientific knowledge about stem cells.

The completion of this chapter, therefore, should be taken as a somewhat convenient provision of a comprehensive

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Table 8.1 Animals that have been used for the harvesting of fats

Domestic animals	Wild animals
Cow and bull	Deer
Sheep	Frog and toad
Pig	Shark
Donkey	Tortoise
Fish	Snake
Cat	Whale
	Mole
	Bear
	Seal
	Elephant

account of the historical and practical utilization of fatty tissues in Chinese Medicine, which could be related to mesenchymal stem cells, and yet is very much different conceptually.

Animal Fats for Therapeutic Uses

The unique nature of Chinese Medicine lies in a most dynamic choice of therapeutic materials. In the case of fat utilization, the choice ranges from the common domestic animals to the rare wildlife type of creatures. Table 8.1 summarizes the animals that have been used for the harvesting of fats.

The following paragraphs give short summaries of the individual items.

Fat of Domestic Animals Used as Therapeutic Agents

Cow and Bull

Acquisition: Direct removal during beef preparation when fresh variety is required or, alternatively, only the oily part is used after cooking and removal of sediments.

Known Chemical Components: Palmitic acid (25–32 %), stearic acid (14–29 %), myristic acid (2–8 %), oleic acid (39–50 %), linoleic acid (1–5 %).

Utilization (General): "Detoxification," hemostasis, and polydipsia. Cow fat is also recommended for skin infections, ulcers, and carbuncles, when used externally as a paste.

Special: A special cooking technique creates a gelatinous budding to be used in patients with polydipsia. Our modern interpretation could be that the nutritive fatty meal replaces the usual carbohydrate rich variety, thus helping to control hyperglycemia and the polydipsia related.

When used externally, a special herbal paste with other ingredients like borneol, mastic, myrrh is prepared [1].

Adverse Effects and Precautions: Avoid over use.

Sheep Fat

Acquisition: Direct removal from sheep or use the oily part after cooking.

Known Chemical Compounds: Mainly glycerin esters, containing palmitic acid (28–29 %), stearic acid (13–15 %), myristic acid (3–4 %), and other unsaturated fatty acids; compared with cow fat, sheep fat appears harder and contains more saturated fatty acids.

Utilization (General): Soothing "toxicity," debilitation, diarrhea, constipation, and polydipsia. When used externally, sheep fat is indicated for cracking skin, burns, frostbites, and skin infections.

Specific: Special formulae for postpartum pain, prolapses, gastric pain, and debilitation [2].

Adverse Effects and Precautions: Contra medicated for those suffering from common cold with cough and sputum.

Pig Fat

Acquisition: Direct removal from pigs or use the oily part after cooking.

Known Chemical Compounds: Resembles both cow and sheep in major components.

Utilization (General): Soothing heat and "detoxification," debilitation, cough, jaundice, and constipation.

When used externally, it is good for cracking skin, burns, and skin infection.

Specific: Known to be good to detoxicate specific poisoning affecting the liver, e.g., related to sulfur.

Special formulation has been created for jaundice, chronic cough, deficiencies, postpartum problems, and constipation [3].

Precautions: Contraindicated for those suffering from diarrhea. Avoid consuming together with plums.

Donkey Fat

Acquisition: Taken from the intra-abdominal cavity of the donkey. The skin of the same animal known as "A-jiu" has been used in traditional medicine as an agent to maintain skin health.

Utilization (General): Detoxification, removal swelling, and controls chronic cough.

Specific: Donkey fat together with black plum is believed to be good for chronic illnesses.

Chronic pain in the limbs could also be served with warmed sheets of donkey fat (apparently fatty omentum) [3].

Precautions: No special description

Fish Oil

Acquisition: Fish oil is available from different types of edible fishes with relatively big sizes. The fish fat is intra-abdominal. Acquisition is accomplished through heating which delivers the oil.

Known Chemical Components: Apart from many fatty acids, fish oil contains Vitamin A and D, phospholipids, cholesterols, etc. The fatty acids usually belong to the unsaturated types.

Utilization: Research on the pharmacological effects of fish oil has been extensive. Following the epidemiological observations that consumers of large quantities of fish oil enjoy better cardiovascular health, more laboratory and clinical studies have confirmed the protective value of fish oil on vascular endothelial integrity, and pharmaceutical companies have already taken much advantage of these observations in their international marketing strategy.

In Traditional Chinese Medicine, fish oil has not enjoyed any priority record. Rather, indications for veterinary uses are seen [4]. In contrast, modern studies on fish oils in China have followed the direction of pharmaceuticals, along the lines of modulating serum cholesterol, thrombotic control, vascular facilitation, and neurological protection.

Precautions: Generally speaking, use of fish oils is considered safe.

Cat Fat

Acquisition: Apparently, it is the Mongolian healers who use the fat of domestic cats for special use. Fat is obtained from the intra-abdominal cavity of cats. Heating produces the oil which solidifies to cake form.

Utilization: Mainly for external uses in burn cases.

The presence in the fat of cats of ganglioside, cerebrosides sphingosines, and phospholipids might have special effects on the regeneration of small vessels [5].

Fat of Wild Animals Used as Therapeutic Agents

Frog and Toad

Acquisition: Wild forest frogs from North East China are considered the best; the exact anatomical site of frog fat lies along the fallopian tubes of the frog. Before winter comes, the female frog stores up a lot of fat in preparation of egg delivery, and this fat is considered a delicacy in Chinese cooking as well as for rejuvenation therapy.

Frog fat is a condensed chain of whitish nodules which do not have any oily appearance. In fact, it is not sticky and even heating does not cause malting.

Utilization: The traditional use of frog fat has been for general rejuvenation – protecting lungs and kidneys; supporting neurasthenia, over sweating, and general weakness.

Modern laboratory tests have confirmed some of these properties so that the physical strength of experimental rats is increased after feeding frog fat for 5 weeks. Other experiments also indicated that frog fat breaks down platelets agglutination and lowers blood cholesterols. Formulations with other herbs have shown antiasthmatic and anti-dementia effects [6].

Deer Oil

Acquisition: Deer oil is taken from both the peripheral and intra-abdominal regions of the deer. Preparation is done by heating the fat to retain the clear oily part to produce light, soft, semitransparent shiny cakes. The best supply comes from Sichuan and Inner-Mongolia.

Utilization: The traditional use is both internal and external. The oral administration is indicated for "detoxification" and driving away the "wind," and swelling. Externally, deer oil is used for dermatitis and painful conditions. It is also used as a fumigant.

Precautions: The traditional use of deer oil is recommended for the male only [7].

Shark Fat

Acquisition: The fat of the shark is taken from the liver of the shark, which is heated to give the oil.

Utilization: Shark fat contains a rich supply of squalene and vitamins A & D as have been analyzed in modern laboratories [8].

Because of the absence of cancer among the sharks, it is believed recently that shark oil might have anticancer effects. In the past decades, therefore, this marine item has been used as an oral agent for the treatment of different types of cancer. Cancers arising from the upper gastrointestinal tract, namely, esophagus and stomach, are believed to the able to get the best benefits.

Tortoise Fat

Acquisition: Tortoise has always been considered a precious animal since this reptile is well known for its longevity. Unlike other animal fats which are prepared for herb-shop

marketing suitable for storage, tortoise fat is obtained from the animal's intra-abdominal cavity and is used fresh.

Utilization: Tortoise fat is used in soup or in other cooking. The indication is for boostering health and energy, both for the male and female.

The oil is also used externally for aging people who start to lose hair and for the purpose of slowing down its rate of turning white. Under such circumstances, tortoise oil is rubbed onto the hair follicles [9].

Snake Fat

Acquisition: The fat is obtained from the large python snake. The fat of the snake occurs subcutaneously and between the muscle strands. Acquisition is therefore obtained through cooking of the sliced up snake. Some snakes give nodular types of subcutaneous fat which is considered particularly valuable.

Utilization: Snake fat is used to control "wind," swellings, skin cracks, and burns. When used externally, the nodular type is painted and pressed onto the diseased skin, either singly or together with other facilitating agent like borneol.

Precaution: It is believed that overuse of the snake oil might cause impotence.

Whale Oil

Acquisition: Whale oil is obtained from both inside and outside the abdominal cavity of the animal. Preparation follows similar procedures described for other animals.

Utilization: The traditional use has been similar to other animal fat. Modern studies have shown that the whale oil could be good for the control of high cholesterol pathologies [10]. Whale oil hence has been combined with standard Chinese herbs that promote circulation, like Salvia for the treatment of coronary diseases.

Oil from Other Rare Animals: Mole, Bear, Seal, and Elephant

Acquisition: The fat of these uncommon animals is obtained from their subcutaneous sites, heated, and then prepared into semisolid cakes.

Utilization: These animal fats share one common indication of use which is general strengthening and detoxification when used as an oral medicinal agent or externally as a skin nutrient.

The fat of the mole has the additional value for pain control and skin sepsis.

Chinese medicinal practitioners have special respect for the big strong animals; hence, the fat from bear and elephant would be given special considerations for strength building.

Conclusion

In any underdeveloped agrarian society, food is important, but food might not be sufficient for the people. When malnutrition is something common, it is not infrequent to see how people value food stuff that would supply energy and replenish bodily energy. Animal fats do have such functions. In the Chinese pharmacopeia, therefore, animal fats are recorded as being "detoxicating" and replenishing.

Although animal fats are widely used as agents to regenerate strength and, often, to counteract debilitation, their use in no way could claim any resemblance to the function of stem cells today. All the animal fats have been used either as a special source of food energizer or externally for skin conditions which include infections and injuries. In the situation of burnt injury and unhealing wounds, one could claim a minor likelihood that fresh animal fats could leave stem-cell-like influences which help to stimulate tissue repair. Apart from that remote speculation, one could conclude that the use of animal fats in Chinese Medicine must have been mainly as a nutritive agent or a topical external applicant.

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Adipose-Derived Stem Cells for Therapeutic Applications

9

Zygmunt Pojda

Introduction

Adipose tissue contains several types of stem and progenitor cells, including the adipose tissue-derived stromal cells (ADSCs), the endothelial progenitor cells, and the hematopoietic and immune system cells. ADSCs share most of phenotypical and functional characteristics of the mesenchymal stromal cell (MSC): the bone marrow-derived mesenchymal stromal cell (BM-MSC) or MSC present in the cord blood, placenta, and umbilical cord. The basic function of ADSC is the preservation of the adipose tissue integrity by the production of adipocytes in the intensity proportional to their degradation. Recently it has been proven that the adipose tissue may contain more MSC-like cells than the bone marrow (which serves as the "gold standard" of cells available for autologous cellular therapies. ADSCs are not only able to differentiate into adipo-, chondro-, or osteogenic lineages but also participate in the formation of the endothelium; smooth, skeletal, or cardiac muscle; hepatocytes; or neural cells. It remains unclear in which extent adipose tissue serves as the natural depository of stem cells, supplying "on-demand" cells for tissue regeneration. ADSCs are the abundant source of autologous stem cells for regenerative medicine techniques, being present in humans throughout all their lifetime.

Adipose Tissue as a Source of Stem Cells

Adipose tissue derives from the mesodermal layer of the embryo [104, 122]. There are several types of adipose tissue, differing in localization and functions: white, mechanical, brown, mammary, and bone marrow. White adipose tissue provides mechanical insulation and energy supply and functions as an endocrine organ, producing the adipokine factors, such as leptin, adiponectin, resistin, osteopontin, lipocalin, and angiogenic-related factors. Mechanical adipose tissue is responsible for more specialized structural support, like palmary fat pads or retro-orbital supporting tissue. Brown adipose tissue plays a unique thermogenic function – being able to generate heat through expression of unique protein – it is localized around the aorta, heart, or kidney in newborn infants, and its volume decreases along with human maturation. Mammary adipose tissue is function specialized, providing the mechanical support and energy for the mammary glands during lactation. The role of the adipose tissue in bone marrow cavities is to replace in adults the space occupied in children by the bone marrow and to provide humoral support (cytokines) and contact regulatory signals for hematopoietic stem and progenitor cells.

Initially, the studies of cells isolated from the adipose tissue were concentrated on adipocytes and their precursors. As early as in 1966, Rodbell and Jones [137–139] were able to isolate the "stromal vascular fraction" (SVF) which was a heterogenous cell population with the predominance of adipocyte progenitors plus the admixture of the fibroblasts, pericytes, and endothelial and blood cells. Consecutive studies [41, 56] revealed that SVF cells have fibroblast-like morphology and are mitotically active source of adipocyte precursors capable to form adipose tissue in vitro. Some authors suggested [45] that under specific conditions, SVF is able to differentiate into non-adipogenic lineages. Almost a decade later, Zhuk et al. [188] demonstrated that the adipose tissue is a source of mesenchymal stromal-type cells (MSCs), capable to differentiate into adipo-, chondro-, myo-, and osteogenic lineages. Subsequently, the same authors demonstrated that the adipose tissue-derived ADSCs express the same marker composition (CD29+ CD44+, CD71+, CD90+, CD105+, SH3+, CD31−, CD34−, CD45−) as the bone marrow-derived mesenchymal stromal cell (BM-MSC) population [187]. The other less numerous population of adipose-derived cells is CD31+, CD34+, CD105+, and

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CD45− and consists of endothelial stem cells (ESCs), characterized by the low expansion rate in vitro [9].

ADSCs are integral component of the adipose tissue, being responsible for continuous replacement of aging adipocytes, resulting in remodeling, and continuous presence of the adipose tissue throughout all lifetime of human being. Several other stem cell populations may derive from the blood vessels (hematopoietic stem cells, immune system cells, or endothelial stem cells) or reside in "stem cell niches" in the adipose tissue following migration from other tissue locations.

Collection and Processing of ADSC

Elective suction-assisted lipectomy (liposuction) has been introduced as a technique of the elimination of the excessive amount of adipose during esthetic medicine treatment (body modification, weight reduction). Liposuction, being one of the basic tools of cosmetic surgery, offers the unique opportunity for collection of large quantities of stem cells from the waste material, without any ethical, medical, or religious contraindications. The same technique may be applied specifically for collection of autologous ADSC for regenerative medicine purposes. Liposuction is not only the less invasive technique as bone marrow aspiration; it allows to collect much higher numbers of cells of MSC characteristics when compared to bone marrow aspiration [24].

The adipose tissue may be obtained by tumescent lipoaspiration [81], ultrasound-assisted lipoaspiration [125], laser-assisted or water-assisted liposuction [2], or surgical resection – all these methods are considered as useful for stem cell collection (in our experience the highest percentage of viable cells is obtained by surgical resection, and the highest, although acceptable, cell mortality results from laser-assisted or ultrasound-assisted procedures). The best results are obtained when the storage time from adipose tissue collection till processing does not exceed 24 h [10].

All the existing protocols for adipose tissue-derived cell separation [8, 54, 123, 188] are based on the enzymatic digestion (collagenase, trypsin) and density gradient separation of ADSCs. Surgical isolation and mechanical dissection of fat, applied in pioneer works [74, 98], was replaced by various liposuction techniques, but all the rest of processing techniques remained basically unchanged. Following lipoaspiration, the mixture of the adipose tissue and balanced salt solution is washed with PBS (purification and removal of anesthetics and epinephrine used during tumescent liposuction) and digested with collagenase. Depending on the technique protocol, cells are isolated by centrifugation, erythrocytes removed by density gradient separation or by addition of erythrocyte lysis buffer, and resulting population of ADSC is expanded in plastic-adherent cultures in media without addition of any growth factors. Cytokine deprivation in in vitro culture allows for further purification of cell population by elimination of residual hematopoietic stem cells originating from blood vessels.

The increasing demand for ADSCs for cell-based therapies resulted in construction of automated systems for adipose-derived cell separation, which can be used at the bedside, without the access to of stem cell laboratory [21, 112]. The advantage of automated devices is (more or less) closed processing system and the possibility of applying cell-based therapies by the groups having no experience in stem cell processing. The disadvantage of automated ADSC processing "on the bedside" is temptation to neglect the verification step of obtained cellular material (tests of cell numbers, viability, phenotype characteristics, etc.) in situations, when cells are isolated by the machine and directly transplanted into patients by surgeons. The other disadvantage of automated system is the cost of the cell isolation procedure and lower flexibility of the procedure when dealing with the material of nontypical quantity or quality.

Phenotypical and Functional Characteristics of ADSC

The procedure of isolation of the adipose tissue-derived cells does not allow to purify the homogenous cell population, resulting in the separation of mixture of mesenchymal stromal cells (MSCs), adipocyte progenitors, fibroblasts, pericytes, and endothelial and blood cells. Such heterogenous population is described by the term "stromal vascular fraction" (SVF) [41, 56, 137–139] or "processed lipoaspirate" (PLA) [118, 188]. The population of adipose tissue-derived stromal cells (ADSCs) is purified by culture in plastic-adherent manner in media non-supplemented with growth factors. Cells which need the supplementation of culture media with growth factors (hematopoietic stem cells) will commit apoptosis, and the more differentiated cells will achieve mature stage and, being nonproliferating, will be eliminated during consecutive passages. The final cell population is composed predominantly of MSC type of cells and is described by various authors as adipose-derived adult cells (ADACs), adipose-derived stem cells (ADSCs, nomenclature advocated by International Fat Applied Technology Society), or adipose mesenchymal stem cells (AdMSCs). Since the "stemness" of adipose-derived cells is not formally proven, the acronym of "adipose-derived stromal cells" (ADSCs) seems most appropriate, reflecting both the adipose tissue origin and mesenchymal stromal characteristics of the cells.

There are several papers discussing the availability of ADSC in comparison with the bone marrow MSC (BM-MSC). The frequency of non-hematopoietic stem cells in human bone marrow, measured by CFU-F assay, varies between 1 in

25,000 and 1 in 100,000 [6, 7, 21, 118]. In contrast, ADSCs are present in frequency of 1 in 50 in population of adipose tissue-isolated nucleated cells [157]. The direct comparison of CFU-F numbers formed by ADSC or BM-MSC plated in the same frequencies of initial cells, revealed the sevenfold higher frequency of ADSC-derived CFU-F in comparison to BM-MSC-derived CFU-F [77]. Based on the frequency of MSC in the bone marrow, and frequency of adipose-derived cells, and on the approximate volume of the adipose tissue or bone marrow collected, it may be concluded that the adipose tissue is a more efficient source for cell collection for therapeutic purposes than the bone marrow [157].

Cell Surface Markers

Phenotypically, ADSCs express surface markers characteristic for MSC category, and, apart from minor differences, their phenotype is similar to BM-MSCs. Both ADSCs and BM-MSCs express markers common for cells having multilineage potential: STRO-1, CD105, and CD166 [26, 47, 48, 103, 128, 155]. The other markers suggesting the therapeutic potential of ADSC are CD29 (beta-1 integrin), important for inducing angiogenesis [4], intercellular adhesion molecule-1 ICAM-1 (CD54) immunoglobulin supergene family [141], and CD44 (hyaluronate receptor involved in development of extracellular matrix) [187]. ADSCs are HLA-DR negative, mostly MHC Class I positive [5], being of low immune reactivity when transplanted in HLA mismatch situation.

ADSCs fulfill the criteria for being multipotential stromal cells, proposed by the International Society for Cellular Therapy (in vitro plastic adherence; expression of CD105, CD73, and CD90 and lack of expression of CD45, CD34, and CD14 or CD11b, CD79a, or CD19 and HLA-DR surface molecules; and capacity of differentiation to osteoblasts, adipocytes, and chondroblasts [30, 57]).

The extended characterization of ADSC surface markers [5, 23, 24, 47, 110, 108, 187] revealed the presence of CD9, CD10, CD13, CD29, CD34, CD44, CD49d, CD49e, CD54, CD55, CD59, CD73, CD90, CD105, CD117, CD146, CD166, and STRO-1 markers and the absence of lineage-specific, hematopoietic, and endothelial markers CD3, CD4, CD11c, CD14, CD15, CD16, CD19, CD31, CD33, CD38, CD45, CD56, CD62p, CD104, and CD144. The expression of VLA-4 (CD49d) and its receptor VCAM-1 (CD106) is reciprocally reversed when comparing ADSC to BM-MSC: ADSCs express CD49d+/CD106- pattern [157], whereas BM-MSCs are CD49d-/CD106+ [23]. The concentration of CD34 marker was higher in freshly isolated cells (SVF) and remained present at reduced levels throughout the culture period of ADSC [110] or have been already unobserved by the others in at least 95 % of cultured cells [77]. Low percentage of CD34-positive cells may reflect the presence of subpopulation of endothelial progenitor cells (EPCs) – the possibility supported by the finding that adipose-derived CD34+ and CD133+ cells are able to form endothelial colonies in vitro or induce angiogenesis in vivo [11, 109, 130, 134, 160]. The concentration of EPC positively correlates with body index, suggesting the entrapment of these cells in the adipose tissue resulting in reduced angiogenic potential in obesity [168].

It has been also documented [15] that ADSCs express Toll-like receptors (TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, and TLR-9) identified both by flow cytometry and real-time PCR. TLRs affect ADSC proliferation and differentiation and play a nonimmune role in signaling on ADSC, but their exact role as structures present on ADSC remains mostly unclear.

Proteome and Transcriptome Analysis

Mass spectrometry analyses revealed the similarities of ASC proteomes and proteomes of fibroblasts and MSCs [25, 161, 172]. Transcriptomes of ADSC and BM-MSC were studied by gene microarrays [75, 99, 175] or Affymetrix gene chips [40]. Both methods have revealed that ADSC and BM-MSC share a common transcriptome [40, 175], expressing stem cell-associated gene markers (Oct4, Sox2, and Rex1) [62].

In Vitro Proliferation and Differentiation of ADSC

ADSCs grow in vitro without supplementation with any growth factors. Fibroblastoid-like cells adhere to plastics and are passaged following trypsinization through a culture period up to 20 passages, or >4 months without visible loss of telomere length [37, 62]. The stable, low senescence level of ADSCs in culture was confirmed by the observation of the absence (<5 %) of β-galactosidase-positive cells in cultures from passage 1 to passage 15 [188]. Data on the telomerase activity are not consistent [40, 62, 75] and may depend on the observation protocols. Cell doubling time varies from 2 to 4 days [62, 110] being longest at the beginning of the culture. In both in vitro and in in vivo animal models, ADSCs are able to differentiate into several "mesenchymal" and "non-mesenchymal" lineages. Since only the minority of experiments were based on the analysis of single cell-derived clonal population of cells [187], the evidence of multilineage differentiation may be assigned rather to the "ADSC cell population" than to the single cell. It has been, nevertheless, proven that ADSC is able to differentiate into other mesenchymal cell lineages – the phenomenon interpreted by some authors as transdifferentiation or plasticity [130, 133, 145, 146].

Differentiation potential of cells residing in the adipose tissue resembles this of the MSC or MSC-like cells residing in the bone marrow (BM-MSC), umbilical cord Wharton jelly (umbilical cord stromal cells, UCSC), cord blood (unrestricted somatic stem cells, USSC), or placenta (reviewed in [131]). It is not surprising that cells resident in the adipose tissue are capable of adipogenic differentiation [28, 51, 144, 151, 187, 188] and, similarly to the other "MSC-type" cells, may differentiate into osteogenic [31, 50, 52, 53, 58, 70, 82, 83, 88, 124, 153, 164, 187, 188] or chondrogenic [32, 35, 128, 174, 175, 187, 188] lineages. The other directions of their differentiation in vitro are myogenic (skeletal muscle [90, 112, 187, 188], smooth muscle [1, 42, 65, 91], and cardiac muscle [44, 129, 156, 158, 179, 187, 188]), neurogenic [4, 71, 86, 142, 145–147, 187], pancreatic [165], and hepatic [152, 162, 163] lineages. It seems to be unclear, if observed angiogenic potential of adipose tissue-derived cells [3, 115, 130, 167] should be attributed to ADSC, EPC, or both cell types, since both are present in adipose tissue and both are capable of endothelial differentiation [130]. It has to be stressed, however, that the majority of experiments describing the differentiation potential of ADSC did not result in the observation of the formation of functional mature cells or tissues but allowed to deduce the differentiation capability from the identification of some structural markers or genetic profiles specific for the cell lineages – so the suggested "differentiation potential" does not mean that ADSCs are able to produce fully functional cells of specific lineage.

Interaction with Hematopoietic and Immune System

The earliest recognized function of mesenchymal stromal cells was formation of "niches" in the bone marrow, where MSC functioned as bone marrow microenvironment, supporting homing and proliferation of hematopoietic stem cells. It has been reported that co-infusion of BM-MSC and hematopoietic stem cells enhanced hematopoietic recovery in chemotherapy-treated patients [84]. ADSCs, being the MSC-type cells, are able to support hematopoiesis in lethally irradiated mice [18]. In intraperitoneal infusion of large quantities (10^7), ADSCs resulted in survival of 40 % of lethally irradiated mice [19], whose hematopoietic cells were of endogenous origin. In all reported experiments, ADSC did not differentiate per se into hematopoietic cells but, similarly to the physiological role of bone marrow MSCs, supported hematopoiesis, playing the role of hematopoietic microenvironment cells.

Mesenchymal stromal cells play an immunomodulatory role when infused into patients with graft-versus-host disease (GVHD) following bone marrow transplantation. It has been observed [87] that in vitro-expanded bone marrow MSCs are able to reduce GVHD symptoms and are efficient in treatment of steroid-resistant GVHD in bone marrow transplanted cancer patients. Comparison of BM-MSC and ADSC revealed similarity in the immunomodulatory properties of both cell types – ADSC did not provoke in vitro alloreactivity of incompatible lymphocytes, suppressed mixed lymphocyte reaction, and suppressed lymphocyte proliferative reaction to mitogens [132]. These findings opened the perspectives for ADSC clinical applications for treatment of patients with severe therapy-resistant GVHD [38, 180].

ADSC and Oncogenesis

There exists evidence on oncogenic potential of bone marrow-derived MSC. MSC may be involved in cancer induction or expansion in several ways – as normal cells supporting cancer growth by migrating towards tumors, modifying tumor environment (vasculogenesis), and immunosuppression or as cells undergoing spontaneous malignant transformation (reviewed in [114]). ADSCs, being a subpopulation belonging to the MSC family, do not differ significantly from BM-MSC in the probability of promotion or induction of carcinogenesis, although the experimental evidence, concerning ADSC role in oncogenesis, is much more scarce than their bone marrow-derived counterparts. Extensive study on the interrelation between ADSC and breast cancer cells [117] revealed that ADSCs are able to home to tumor site even when injected intravenously and incorporate into tumor vessels, where they differentiate into endothelial cells. Direct contact of ADSCs with tumor cells results in enhancement of secretion from ADSCs of stromal cell-derived factor 1 (SCF-1), which acts in a paracrine fashion on the cancer cells enhancing their motility, invasion, and metastases. It has been also documented that ADSCs, similarly to their interactions with breast cancer, were recruited towards cancer cells through SDF1/CXCR4 axis and supported cancer growth by increasing tumor vascularity when cocultured with prostate cancer cells in athymic mice [97].

Standard ex vivo expansion procedure, when ADSCs are cultured for 6–8 weeks, is "safe" and does not lead to the phase of cell transformation events. It has been documented, however, [143], that after in vitro expansion lasting 4–5 months, human or mouse ADSC spontaneously bypassed the senescence and crisis phase, showing altered phenotype and chromosome instability and losing contact inhibition capacity. At this stage, cells were able to induce cancer when injected into immunodeficient mice. The general conclusion from the observations on long-term expansion of ADSC is that the cells, expanded "traditionally" for the period of 6–8 weeks, may be considered as a valuable tool for tissue regeneration and engineering, but the prolonged in vitro culture may cause the risk of spontaneous

transformation and induction of cancerogenesis in transplant host [143]. Contrary to the observations of the immortalization of ADSC, after prolonged in vitro culture, the aberrant, tumorigenic cell line was isolated as early as from third-passage cells [121] – the result suggesting the need of rigorous testing of in vitro-expanded ADSCs prior to their clinical applications.

Clinical Applications of ADSC

Subcutaneous Tissue Formation

The adipose tissue is present physiologically in multiple locations in human body, being responsible for multiple functions (mechanical, endocrine, thermoinsulatory, and energy supplying). Typical surgical procedures (liposuction, lipotransfer) are performed for cosmetic rather than medical purposes – the exemption is the application of lipotransfer technique for treatment of breast cancer patients after mastectomy, where injection of the adipose tissue not only partially reconstructs the amputated breast but locally supports better healing and prevents formation of connective tissue scar between the skin and muscles. Enrichment of lipotransferred autologous adipose tissue with ADSC isolated from the same patient [105, 184] reduces the atrophy of implanted tissue and supports the formation of new adipocytes in the region of implantation. Immunosuppressive potential of implanted ADSC may also minimize the inflammatory reaction in the implantation area. There is some consideration [101] if the implantation of ADSCs may increase the risk of cancer recurrence; however, such speculations seem to be not substantiated by the observations. Similar technique of ADSC enrichment of implanted adipose tissue was used for corrective treatment after artificial breast implants removal caused by various complications (like capsular contracture), and the results were described as satisfactory [185]. As a support and a carrier for transplanted ADSCs, "injectable scaffolds" consisting of cell-binding polyglycolic acid (PGA) [14], poly (lactic-co-glycolic acid) or PLGA [127], hyaluronic acid [49], fibrin [149], matrigel [76], or alginate gel [182] are applied. The in vivo study has shown that ADSCs attached to micronized acellular dermal matrix (Alloderm) and cultured for 14 days in adipogenic differentiation media were able to differentiate into mature adipocytes when implanted subcutaneously into dorsal cranial region of nude mice [183]. For the applications, when the elastic, mechanically resistant, non-immunogenic, and slow degradable scaffold is needed, 3-D scaffolds of silk fibroin were developed [106]. Interesting, although not yet validated, is the exploitation of the ability of ADSC to produce a variety of growth factors, regulatory factors, and collagen for skin antiaging therapy [126].

Bone Formation

The bone formation phenomenon was observed prior to the experiments with ADSC differentiation, in patients with progressive osseous heteroplasia, which is characterized by spontaneous formation of calcified nodules in the adipose tissue [72, 154]. In vitro, both human and animal ADSCs may be stimulated to differentiate into osteogenic lineage [28, 51, 144, 151, 187, 188], producing cells of osteogenic phenotype characterized by the presence of bone markers: alkaline phosphatase, osteopontin, osteonectin, type I collagen, bone sialoprotein, osteocalcin, BMP-2, BMP-4, and BMP receptors I and II. In vivo ADSCs differentiate into the bone when implanted ectopically into rodents [55]: rat-isolated ADSCs, seeded in polyglycolic acid, form the bone when implanted subcutaneously [89]. Similarly, human ADSCs in HA-TCP scaffolds differentiate to osteocytes in immunodeficient mice [31, 33]. ADSCs, when seeded in apatite-coated PLGA scaffolds and surgically implanted, were able to repair surgically created critical-size calvarial defects in mice [20]. In contrary to these observations, poly-L-lactic scaffolds colonized with non-differentiated ADSCs were unable to repair experimental rat palatal bone defects, while similar implants containing osteogenically differentiated cells fully reconstructed the bone defects in vivo [17]. Basing on these in vivo experiments, ADSCs were collected from a 7-year-old girl with large, bilateral calvarial defect, combined with iliac crest bone fragments and fibrin glue on resorbable mesh, and autologously implanted, treatment resulting in marked ossification and regeneration of defect to near-complete continuity after 3 months following surgery [92].

Cardiac Repair and Angiogenesis

Morbidity and mortality, resulting from cardiovascular diseases (CVDs), account for approximately 30 % causes of deaths, constituting major medical, social, and economical problem. At the beginning, the rationale of stem cell therapy of cardiac infarct was to implant cells, which will be able to transdifferentiate into cardiomyocytes and regenerate the necrotic region of the cardiac muscle. The obvious candidates, according to the cell plasticity concept, were hematopoietic stem cells from the bone marrow or umbilical cord blood. The effects observed in animal experiments were the increase of muscle mass in regenerating heart muscle, improvement of cardiac hemodynamics, and, surprisingly, very low frequency of the presence of myocardial cells of donor origin. Detection in the adipose tissue of the MSC-type cells capable of myogenic differentiation resulted in in vivo experiments based on intracardiac transplantation of ADSC in models of coronary disease or myocardial infarction [170]. It has to be determined, if the beneficial effects of

treatment with ADSC results from differentiation of ADSC into myocardium or in paracrine mechanisms supporting endocrine repair [11, 13, 109, 119, 134, 156, 158, 169]. In 2004 the cardiomyogenic potential of ADSC has been documented [130, 175]; since then multiple studies have confirmed the phenomenon of direct formation of cardiac muscle by ADSCs [111, 173, 186]. It has been shown [100, 179] that the brown adipose is the best source of cells capable of cardiomyocyte differentiation. Treatment with ADSCs significantly improves functional parameters of regenerating heart, such as neovascularization [12, 13, 109, 130, 186], collateral perfusion [66, 67], and hemodynamic parameters (ventricular end-diastolic dimension, ejection fraction, cardiac output) [22, 107, 148, 169, 173]. ADSC transplantation into the heart does not increase arrhythmogenic tendency of the cardiac muscle [39, 73]. Some improvements may result from secretion humoral factors (angiogenic cytokines) by ADSC [134] or direct formation of endothelium and, in consequence, angiogenesis [12, 13].

The same mechanisms allow using ADSC for treatment of animal model of severe hind limb ischemia [119, 134]. Considering the importance of treatment of cardiac ischemia and infarct and the beneficial effects of ADSC on cardiac muscle regeneration, there is a real possibility of expanding the role of autologous ADSC in cardiac muscle regeneration and treatment of diseases with ischemic background.

Cartilage Repair

In general, the diseases originating from cartilage defect, resulting from injury, autoimmunity, or degenerative disease (osteoarthritis), have strong negative impact both at the patient's level and at the social and economical levels. There have been published several attempts of inducing of cartilage repair using autologous stem and progenitor cells. In young patients with isolated cartilage lesions, the use of culture-expanded autologous chondrocytes seems most promising. In elderly patients, suffering from the massive denudation of articular cartilage, the availability of autologous expanded chondrocytes is, however, reduced and insufficient for therapy, so there is demand for another autologous cell source. The candidate cells must be available in adult donor, and their collection must be safe and relatively uncomplicated; these cells must have the potential for differentiation into chondrogenic lineage both in vitro and in vivo. Such cells must be also available in patients with osteochondral defects, so the original disease must not influence the numbers and qualities of cells collected for treatment. The candidate cells, fulfilling the criteria, are ADSCs collected from patient's adipose tissue [120]. Comparison of the chondrogenic potential of BM-MSCs and ADSCs isolated from various locations confirmed that all these cells are able to differentiate into chondrocytes in vitro, but their differentiation potential depends on the source [113, 171, 177]. Some authors claim the superiority of ADSC over BM-MSC [24], but prevailing data suggest that BM-MSCs have superior chondrogenic potential when compared with ADSC [16, 59, 61, 85, 99, 126, 135, 150, 175]. The exception is intrapatellar fat pad, which is a much better cell source than subcutaneous adipose tissue [34, 113]. The future of ADSC as a candidate for cellular repair of cartilage is unclear; some findings suggest that improvement in in vitro/in vivo stimulation of chondrogenic differentiation of ADSC may increase their importance as candidates for clinical applications [36, 78].

Central Nervous System Repair and Regeneration

Limited natural capacity of self-renewal of neural system, combined with high frequency of accidents and diseases resulting in neural system dysfunction, emphasizes the importance of development of the new methods for stem cell application in neurological disorders. ADSC is capable of differentiating into neuropoietic lineage as well as regulating the neural repair and restoration of local circulation in central nervous system. Several authors documented that ADSCs are able to differentiate in vitro into neural cells [32, 60, 64, 71, 99, 181, 187], interact with neural cells on paracrine level [68], or produce Schwann-type cells [178]. There exists no evidence that adipose-derived cells, differentiating into neural cells, derive from the neural crest lineage [176].

There is also, unfortunately, no evidence that so-called neural cells observed in vitro are indeed mature functional neural cells – most authors recognize cells of "neural morphology" after identification selected markers present on early neural cells, like microtubule-associated protein, neuronal nuclear antigen, β-tubulin III [60], neurofilament 1 (NF1), nestin, neuron-specific enolase (NSE) [181], or neurosphere formation [71]. The other data derive from in vivo animal experiments, where ADSCs are implanted into regions of injury of neural system. The intensively researched problem is the possibility of amelioration of brain stroke effects by local application of ADSCs. Possible therapeutic effects may result from direct replacement of ischemia-eliminated brain cells, regulation of neural cell regeneration in paracrine manner, or reconstitution of local microcirculation by angiogenesis mediated or formed by ADSCs. When human ADSCs were injected into lateral ventricle of healthy rats, they were able to migrate to multiple areas including the contralateral cortex and could be locally identified up to 30 days following implantation. Similar implantation of ADSCs into the brain 1 day after MCA occlusion (the experimental model of stroke) resulted in cell migration into the ischemic area and localization at the border between the

intact and injured brain tissue [69]. Injection of ADSCs did not change the infarct size but significantly improved the recovery in motor and somatosensory behavior aspects, suggesting that at least there exists the mechanism of local trophic support from ADSCs [69]. In other experiment, ADSCs not only improved neurological functions of infarcted rats but also markedly attenuated brain infarct size [93]. Immunomodulatory effect of ADSCs was exploited in the compassionate study on three patients with multiple sclerosis (the disease caused by the autoimmunity mechanisms). Multiple intravenous or intrathecal infusions of autologous ADSCs, combined with allogeneic CD34+ and MSCs, resulted in marked improvement in disease status of all patients, although the observation is very preliminary and statistically not significant [136]. ADSCs were tested for their ability to accelerate the spinal cord fusion (treatment for lumbar compression fractures) in rat model. Local application of scaffolds colonized by autologous or allogeneic ADSCs into the injury site reduced inflammatory cell infiltration and accelerated posterior spinal fusion process [102]. ADSC may act through the different mechanisms, like local regulation by paracrine manner [80, 94], participation in local angiogenesis, or immunomodulatory effects; the phenomenon of direct ADSCs differentiation to neural cells cannot also be excluded. Nevertheless of the mechanisms of ADSC actions, the preliminary in vivo results suggest the usefulness of both autologous and allogeneic ADSCs in treatment of central nervous system diseases and injuries.

Other Therapeutical Applications of ADSC

Experimental and clinical applications of ADSC resemble those exploited earlier with the use of BM-MSC. ADSCs seem to be the cell population, which may be widely used for gene therapy. In autologous transplantation model, gene-transfected ADSC guarantees relatively high safety, and their reported ability to maintain stable telomere length [37, 62] and long proliferation time in in vitro systems guarantees long-term delivery of gene product. Parallel experiments with infection of both MSCs and ADSCs with E1A-deleted type 5 adenovirus constructs containing the BMP-2 (bone morphogenic protein-2) gene or the bacterial beta-galactosidase (lacZ) gene resulted in 55 % transduction efficiency for ADSC in comparison with 35 % efficiency for BM-MSC [31], which resulted in threefold higher expression of BMP2 protein by ADSCs than by BM-MSCs. Experiments on stability of lentiviral vector-transduced cells revealed the presence of transduced cells in culture over 100 days at transduction efficiency of 98 % [116].

There are rather scarce data on the differentiation of ADSC into several cells and tissues, like skeletal and smooth muscle, hepatocyte-like cells, or pancreas endocrine cells. When transplanted into mdx mice (murine model of Duchenne muscular dystrophy), ADSC helped to regenerate the muscle and induced expression of dystrophin [140], although their role in muscle repair is still rather unclear. In vitro, ADSC differentiates into cells of myogenic phenotype, resembling the characteristics of skeletal muscle, the process observed when ADSCs are directly contacting primary muscle cells [27, 90]. Observations of in vitro capacity of ADSC to differentiate into smooth muscle cells [1, 42, 65, 91] were clinically exploited in attempted urinary incontinence treatment and bladder reconstitution [63], with results not substantially different to those obtained when used BM-MSC. There exist a scarce data on hepatopoietic differentiation potential of ADSC. In vitro, ADSC cultures in the presence of HGF, OSM, and DMSO form cells of hepatocyte-like phenotype, expressing albumin and α-fetoprotein, capable to take up low-density lipoprotein and to produce urea [152]. Following these observations, ADSCs, intravenously injected into mice, were detected in injured liver, and their integration into the liver was augmented by partial hepatectomy [79]. Preliminary data confirm the ability of ADSC to differentiate into cells of pancreatic endocrine phenotype partially maintaining pancreatic endocrine cell functions. Following the stimulation with activin-A, extendin-4, HGF, and pentagastrin, cells expressed pancreatic endocrine transcription factor Isl-1; developmental transcription factors Pax-6, Ipf-1, Ngn-3; and expressed pancreatic hormones insulin, glucagon, and somatostatin [165]. The data are too preliminary and need to be extended and confirmed, but even now they give some hope for the use of ADSC for cell-based therapy for type 1 diabetes mellitus. There exist also several reports of preliminary results after ADSC treatment of such varying diseases as Crohn's disease (occlusion of rectovaginal fistula) [43, 46], wound healing [95], erectile dysfunction [96], tissue engineering (bypass graft construction [29], production of skin substitutes [166]), or feeder layer for induced pluripotent stem cells (iPSCs) [159]. All these reported ADSC therapeutic applications have one common characteristic – they need much more research for data collection and validation before their potential usefulness may be evaluated.

Conclusions

The phenotype, functional characteristics, and differentiation potential of ADSC are enough similar to their BM-MSC counterparts to conclude that the differences between ADSC and MSC are not important in the aspects of their applications for cellular therapy. The advantages of ADSC over MSC lay in the possibility of collection of much larger numbers of cells without endangering patient's health. The other advantage is higher purity of isolated ADSC population – bone marrow aspirates consist of much higher numbers of hematopoietic cells than MSCs, and the most efficient method of primitive

BM-MSC isolation (bone grinding) is impossible to use when considering collection from living donor. The most promising clinical applications of ADSC, according to presently available data, are treatment of cardiac ischemia and myocardial infarction, central nervous system repair following accidents or stroke, treatment of immunology-related diseases (graft-versus-host disease, multiple sclerosis), and techniques of bone and joints replacement and repair using scaffolds seeded with ADSCs and their more differentiated progeny. In the esthetic medicine/plastic surgery, ADSCs are the "cells of choice" for corrections of irregularities in subcutaneous tissue distribution.

In general, availability of large numbers of autologous cells in any patient's age, safe protocols of cell collection, in vitro expansion and differentiation, multilineage differentiation potential, and in vivo immunomodulatory capacity make ADSC the almost ideal cell type for cellular therapy, gene therapy, and regenerative medicine.

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Subcutaneous Adipose Tissue-Derived Stem Cells: Advancement and Applications in Regenerative Medicine

10

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Abbreviations

ADAS	Adipose-derived adult stem cells
AdMSCs	Adipose mesenchymal stem cells
AIT	Autologous islet transplantation
ASCs	Adipose-derived stem cells
AutoHS	Autologous human serum
BAT	Brown adipose tissue
BM	Bone marrow
BMI	Body mass index
BMP	Bone morphogenetic protein
BMSC	Bone marrow stem cell
BSA	Bovine serum albumin
CAL	Cell-assisted lipotransfer
CAM	Cell adhesion molecule
CCl4	Carbon tetrachloride
CD	Cluster of differentiation
CNS	Central nervous system
DEX	Dexamethasone
DMD	Duchenne muscular dystrophy
DMEM-HG	Dulbecco's Modified Eagle's Medium–high glucose
DMEML-G	Dulbecco's Modified Eagle's Medium–low glucose
EBP	Enhancer-binding protein
ECM	Extracellular matrix
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
GGF	Glial growth factor
GPDH	Glycerol-3-phosphate dehydrogenase
GVHD	Graft-versus-host disease
HA-TCP	Hydroxyapatite/ tricalcium phosphate
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
hMADS	Multipotent adipose-derived stem cells
IBMX	3-isobutyl-1-methylxanthine
IFATS	International Fat Applied Technology Society
IFN	Interferon
ISCT	International Society for Cellular Therapy
MEM	Minimal essential media
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
PDGF	Platelet-derived growth factor
PLA	Processed lipoaspirate
PLGA	Poly(lactic-*co*-glycolic acid)
PPARγ, LPL	Peroxisome proliferator-activated receptor γ, lipoprotein lipase
PTH	Parathyroid hormone
SF	Subcutaneous fat
SLE	Systemic lupus erythematosus
SVF	Stromal vascular fraction
TGF	Transforming growth factor

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TTR	Transthyretin
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WAT	White adipose tissue

Introduction

Stem cell research has been hailed for its potential to revolutionize the field of regenerative medicine with the ability to regenerate damaged and diseased organs. In addition to offering unprecedented hope in treating many debilitating diseases, stem cells have advanced our understanding of basic biological processes. Intense study on stem cells in the past decade has kindled worthy knowledge about developmental, morphological, and physiological processes that form the basis of tissue and organ formation, maintenance, repair, and regeneration. Today's medicine generally tries to support or treat injured tissues and organs, but stem cells simply replace them [1]. Stem cell research is complicated and rapidly changing. The correlation of stem cell technology with tissue repair still has a long way to go. Since embryonic stem cells are a thorn inside when it comes to the ethics of therapeutics, stem cells isolated from adult tissues sidestep this issue entirely and have become a potent contemporary source of stem cells for tissue repair and regeneration. Conceptually and from a practical standpoint, the bone marrow has been the most influential source of stem cells that offers a possibility of being used in a wide range of therapeutics. Clinical situations frequently demand stem cells with dependable quality and quantity to treat disorders of cellular degeneration. Challenges to bring advances to the clinical mount have expanded rapidly, engendering new perspectives concerning the identity, origin, and full therapeutic potential of various tissue-specific stem cells.

Recent progress in stem cell biology has allowed researchers to investigate distinct stem cell populations in such divergent mammalian tissues and organs such as the tendon [2], periodontal ligament [3], synovial membrane [4], lung [5], liver [6], endometrial tissue [7], and body/tissue fluids such as synovial fluid [8], amniotic fluid [9–11], and menstrual blood [12, 13]. Regardless of the ubiquitous presence of stem cells, taking those stem cells adaptable for regenerative medicine applications in adequate quantities at the right time is a challenge. In this respect, an emerging body of literature suggests that redundant adipose tissue serves as an abundant, accessible, and reliable source of stem cells that can be readily harvested with minimal risk to the patients. Rapidly accumulating evidence suggests that adipose tissue-derived stem cells (ASCs), especially from white adipose tissue, possess a far wider property of self-renewal and multilineage differentiation capacity, thereby highlighting their importance and effectiveness in regenerative medicine [14–18]. Despite literature supporting the plasticity of adipose-derived stem cells for regenerative medicine, there are functional and heterogenous discrepancies associated with it, thus presenting ASC research a difficult and challenging task. Promising strides are continuously being made to unravel these challenges and realize the potential of ASC. While much progress on adipose-derived stem cells has been made in the last few years, there remain a lot to be explored.

This chapter, on the front line, focuses on the overview of current status of knowledge on stem cells derived from white adipose tissue, particularly from subcutaneous fat, representing the salient features of its unique characteristic attributes, viz., isolation and extensive expansion, multilineage differentiation, phenotypic characterization, and media optimization. It also attempts to review the advances that have occurred in recent years in the applications of adipose tissue-derived stem cell technologies in regenerative medicine along with the future promises of the "holy grail" of medicine.

Classification of Adipose Tissue

Adipose tissue is a highly specialized and complex connective tissue that is present in all mammalian species and a variety of nonmammalian species. The primary function of the adipose tissue is limited to the contribution of energy storage in the form of fat. It plays a central role in energy homeostasis, through a network of endocrine, paracrine, and autocrine signals, and has been identified as a highly active metabolic organ [19]. Adipose tissue secretes factors that influence the endocrine regulation of the body, affecting growth, metabolism, and behavior. It exhibits different properties according to its anatomical localization. Functional difference in adipose tissue seems associated with the regional distribution of fat depots, such as white adipose tissue (WAT) and brown adipose tissue (BAT). Despite the difference in functions of both the tissues, they are both named as adipose because of their triglyceride deposits [20].

Brown adipose tissue (BAT), as the name suggests, is brown in color due to the higher cytochrome oxidase content of its mitochondria, which are abundantly found in the cytoplasm. Anatomical distribution of BAT differs from that of WAT. Brown adipose tissue (BAT) is predominantly located in newborns and in hibernating mammals and acts as the primary heat source at a young age. During the natural aging process, brown adipose tissue is gradually replaced by white adipose tissue which is used as a substrate, or starting material, from which energy is generated by a series of biochemical reactions. In contrast, brown adipose tissue, rich in blood vessels and mitochondria, yields energy directly, without intervening chemical breakdown reactions [21]. The cells of brown adipose tissue may be polygonal or ellipsoidal in shape, with a cellular diameter ranging between 15 and 50 μm. BAT contains several smaller lipid vacuoles and a high number of mitochondria that are widely distributed in the cytoplasm and differ in size, giving brown adipocytes a

multilocular appearance [22]. Thus, brown and white adipocytes can be distinguished from each other by size and morphology, abundance of mitochondria, and, most importantly, the presence of uncoupling protein 1 (UCP1) [23]. BAT produces thermogenesis that is spread throughout the body via blood circulation. The regulation of thermogenesis is mainly controlled by the hypothalamus; the sympathetic system carries the signals and releases norepinephrine, which induces fatty acid metabolism, in the mitochondria of brown adipocytes [24]. Heat production is accomplished by the action of a special protein in the inner membrane of the mitochondrion, UCP1, unique to BAT. Brown and white adipose tissues are both innervated by the sympathetic nervous system, which controls metabolic and lipolytic activity as well as the vascularization in adipocytes.

White adipose tissue (WAT) is the only tissue in the body that can markedly change its mass after adult size is reached. In humans, the normal white adipose tissue mass composes 9–18 % in males and 14–28 % in females. In obesity, fat mass exceeds 22 % of the body weight in males and 32 % in females. However, the fat mass can vary depending on the individual ranging from 2 to 3 % of body weight in extremely well-conditioned athletes to even 60–70 % of body weight in massively obese individuals [25]. White adipose tissue is located primarily in three major anatomical areas – subcutaneous (inguinal, dorsal subcutaneous aka axillary and interscapular fat depots), dermal (a relatively continuous sheath of lipids), and intraperitoneal (mesenteric, omental, perirenal, retroperitoneal, epididymal, and parametrial) fat depots. The cells are round or polygonal, ranging between 25 and 200 μm in size. The WAT adipocyte contains a single large lipid droplet in each cell. This droplet does not have a well-defined limiting unit, but has a monolayer membrane between the intracellular lipid component and the cytoplasm [26]. Although these cells contain many organelles, their recognition becomes difficult as the large lipid droplet pushes the organelle, including the nucleus, towards the thin cytoplasm under the plasmalemma. During routine histological processing, lipid becomes dissolved, leaving an empty space that can be seen as a typical signet ring shape under a light microscope [27, 28]. WAT basically functions as (1) energy source for the body along with heat insulation and cushioning, (2) shock absorber on the basis of its anatomical location, (3) has endocrine functions, (4) fills body spaces, and (5) helps lubricate neighboring muscles to allow ease of movement.

Components of White Adipose Tissue

The primary cellular components of white adipose tissue are the so-called adipocytes or lipid-filled fat cells. A population of mature adipocytes contains fat cells of variable size, including very small fat cells (<3.5 μm diameter) [29]. Interestingly, as the population of fat cells increases by diameter or volume with growth or the development of obesity, the heterogeneity of the cell population diminishes. This suggests a maximal adipocyte size that may vary from species to species and from depot to depot. White mature adipocytes consist of about 90 % lipids and each of them stands in close contact with at least one capillary, providing a vascular network that allows continued growth of the organ.

A further integral component of white adipose tissue, apart from the adipocytes, are the stromal cells, identified as stromal vascular fraction (SVF) which is mainly located around the blood vessels. SVF was identified to contain a large population of preadipocytes, first identified by Poznanski et al. in 1971 [30], following enzymatic digestion of adipose tissue. In addition, these harvested adipose stromal vascular fractions were found to possess heterogenous cell population of cells from the microvasculature, such as vascular endothelial cells and their progenitors, pericytes, myeloid dendritic cells, nerve tissue, loose connective tissue matrix, cells present in stroma, and ECM including fibroblast, vascular smooth muscle cells, mesenchymal stem cells, and immune cells such as resident hematopoietic progenitor cells and macrophages [31–33]. Additionally, SVF also possesses leukocytes that may be resident in the parenchyma of adipose tissue [33]. Despite the fact that SVF is a heterogenous cell population, subsequent expansion of the SVF in vitro selects for a homogenous cell population.

Dynamics of White Adipose Tissue

White adipose tissue has a remarkable ability to undergo considerable changes in volume during the life span of an individual. The cellular development associated with relatively small increases in volume can be accommodated by changes in the amount of lipid stored in individual adipocytes (cellular hypertrophy), and larger changes are mediated by the generation of new adipocytes (cellular hyperplasia) accompanied by coordinated expansion and remodeling of the adipose vasculature [26, 34]. Hypertrophy is the result of excess triglyceride accumulation in existing adipocytes due to a positive energy balance. Hyperplasia, or "adipogenesis," involves the recruitment of new adipocytes from precursor cell component in adipose tissue via proliferation and differentiation of preadipocytes. These changes of adipogenesis are mediated first by the proliferation of a population of stem/progenitor cells that are located within the stromal vascular fraction of the adipose tissue to form preadipocytes and second by the process of differentiation, by the form of transition from undifferentiated fibroblast-like preadipocytes into mature round lipid-filled fat cells, characterized by the unilocular appearance of the mature fat cell [35, 36].

These precursor cells are believed to be present throughout the adult life for continuous synthesis of new adipocytes [37]. Thus, from early days, researchers have studied the adipogenic potential of these stem cells using preadipocyte primary

culture (derived from stromal vascular fraction of adipose tissue from various species) and established cell lines of murine origin, committed to adipocyte lineage [38–41], for various reasons: firstly, white adipose tissue is a key player in metabolic homeostasis through its role as both an energy depot and endocrine organ, and secondly, excess adiposity and adipose tissue heterogeneity in obesity are associated with impaired lipid and glucose homeostasis, leading to hyperglycemia, insulin resistance, and type 2 diabetes. Research done so far on the dynamics of WAT, too, indicates that recruitment of lipid-rich preadipocytes from the SVF is done far more rapidly at WAT deposit areas, contributing to the metabolic syndrome. The bulk of the WAT is broadly divided into two main localizations – subcutaneous fat and mesenteric fat, best represented by omentum fat. New insights on WAT biology emerged when the redundant subcutaneous fat, being more abundant and easily accessible, was identified to be the major reservoir of stem cells of potential use for cell-based therapies.

Subcutaneous Adipose Tissue-Derived Stem Cells

Subcutaneous fat is found just below the skin in a region called the hypodermis and intramuscular fat which is found interspersed in skeletal muscle. Like all other fat organs, subcutaneous fat is an active part of the endocrine system, secreting the hormones leptin and resistin. Typical female pattern of body fat distribution around the hips, thighs, and buttocks is subcutaneous fat and therefore poses less of a health risk compared to visceral or the omentum fat. This subcutaneous fat is not related to many of the classic obesity-related pathologies, such as heart disease, cancer, and stroke, and there is even some evidence that it might be protective. Subcutaneous adipose depots are accessible, abundant, and replenishing, thereby providing a potential source as adult stem cell reservoir for each individual. Until the identification of stem cells from omentum fat, the term adipose-derived stem cell (ASC) was the terminology commonly used to refer the stem cells of subcutaneous fat alone. Attention in considering subcutaneous adipose tissue as a reservoir of stem cells was really undertaken only after the findings of Zuk and his co-workers, in the year 2001 [18, 42]. The translation of his findings associated with the easy sampling of adipose tissue with its low risk and morbidity attracted many new investigators. Subsequently, increasing evidence is accumulating on the pivotal role of subcutaneous fat-derived stem cells owing to their proliferative capacity and multilineage differentiation ability [16, 17, 43–47].

In a wide perspective, a range of names has been used to describe the adherent cell population isolated from adipose tissue, e.g., lipoblast, preadipocytes, processed lipoaspirate (PLA) cells, adipose-derived stem/stromal cells (ASCs), adipose-derived adult stem (ADAS) cells, adipose-derived adult stromal cells, human multipotent adipose-derived stem cells (hMADS), and adipose mesenchymal stem cells (AdMSCs) [48, 49]. To address the problem, a consensus reached by investigators at the 2004 conference of the International Fat Applied Technology Society (IFATS) in Pittsburgh has proposed a standard nomenclature by adopting the term ASCs to identify the isolated, plastic-adherent, multipotent cell population [33].

Isolation of Subcutaneous Adipose Stem Cells

In humans, the stromal vascular fraction can be isolated from the redundant subcutaneous adipose tissue, in several regions of the body, especially the abdomen, either by using liposuction aspirate or during reconstructive surgeries like the "tummy tuck" or abdominoplasty. When the starting material is obtained from liposuction procedure, the isolation method is simplified, as the procedure generates finely minced tissue fragments that are more homogenous, allowing a more efficient enzymatic digestion. When working with the solid tissue pieces as starting material, the tissue is minced manually, requiring more time and effort for thorough enzymatic digestion. In 1964, Martin Rodbell was the first to present a method for in vitro isolation of mature adipocytes and adipogenic progenitors from fat tissue obtained from rat [50]. The material is washed after harvesting to remove possible blood contamination. The tissues are minced into small fragments and the extracellular matrix (ECM) holding the adipocytes in place is digested with type I collagenase solution at 37 °C, followed by centrifugation that separates non-buoyant stromal cells from the buoyant adipocytes.

Following this, adipose stem cell research began in 1992 [51], when investigators used cultures of stromal vascular cells isolated from porcine preperitoneal fat in media with heparin and endothelial growth factor, which, they reported, had similar morphology to human subcutaneous adipose cells. This produced cells that stained positive for von Willebrand factor (vWF), α-smooth muscle cell actin, and cytokeratin and were termed as microvascular endothelial cells. Subsequently, the stromal vascular fraction has been shown to include multipotent mesenchymal stem cells, which may reside in the perivascular region of the stroma. Zuk and co-workers were the first to show that the SVF fraction isolated from human liposuction samples contained cells with multilineage potential and termed as processed lipoaspirate (PLA) cells [18, 42]. They allowed the ASCs to adhere to the plastic surface of tissue culture flasks, which is still the basis of most methods used to date. Since then, several groups working independently have developed and refined procedures of isolating and characterizing adipose-derived stem cells obtained from both lipoaspirates and the excised solid fat tissue obtained through lipectomy [18, 35, 43, 53, 54].

The yield of stromal vascular fraction obtained using different harvesting techniques such as solid fat tissue through reconstructive lipectomy surgeries, tumescent or conventional liposuction, and ultrasound-assisted liposuction and harvesting sites, such as subcutaneous fat of the abdomen and hip/thigh, has also been investigated. Fraser and his co-workers contended that neither the site of harvest nor the harvesting technique affected the number of stem cells obtained [52]. On the contrary, Varma and his co-workers suggested that the harvesting technique affected the recovery of ASCs, with ultrasound-assisted liposuction yielding the lowest number of proliferative ASCs [53]. Later, the same group and other researchers also identified that the site of harvest affects the yield of ASCs. They reported that frequency of cells in abdominal subcutaneous fat is much higher than the frequency obtained in hip/thigh subcutaneous fat [43, 53, 54]. In addition, Varma and his co-workers also reported that SVF cells derived from abdominal fat reach 80–90 % confluency within 5 days, whereas SVF cells derived from adipose tissue of the hip/thigh take more than 9 days to reach 80–90 % confluency when seeded in the same density [43]. To conclude, it is probable that subcutaneous fat obtained from the abdomen for SVF isolation is the better harvest site when compared to the hip/thigh.

Yield comparison derived from lipoaspirates as well as from lipectomy identified from the subsequent work suggested that abundant number of ASCs can be derived from lipoaspirates as well as from lipectomy procedure. The non-buoyant stromal cells were identified to yield in excess of $1\times10^6-6\times10^8$ ASCs from stem cells from 100 to 300 cc of abdomen fat in comparison to 2.5×10^4 stem cells from the 40 cc of bone marrow aspiration [14, 18, 45, 54–56], with more than 90 % of the cells being viable. Owing to the greater concentration of cells from subcutaneous fat and its favorable characteristics, it was reported that the ASCs can directly be used in different therapeutic doses for treating a variety of diseases without any further expansion in culture [49, 57–60].

Phenotypic Characterization of Subcutaneous ASCs

Despite nearly a decade of research efforts, the phenotypic and functional characteristics of ASCs remain obscure. Although fibroblastic morphology of cells is one of the characteristics to identify MSC, such characterization alone is insufficient to confirm the isolation of a true ASC population. Thus, immunophenotypic characterization of cell surface markers becomes an ideal characteristic feature of stem cell. It is uncommon to find a protein whose expression is completely specific to one particular cell type and is therefore usually necessary to build an expression profile which considers a range of markers known to be expressed by a given cell type. Many attempts have been made to develop a cell surface antigen profile, in order to better purify and identify ASCs, especially a common immunophenotype which could enable isolation of a purified population of ASC. However, to date, no single marker has been identified that delineates ASC in vivo, and hence, there is a lack of thorough understanding of the mechanism underlying stem cell renewal and its functional differentiation. Thus, quest for the identification of a prospective definitive biomarker remains elusive. The simple way of identification is from the progressive loss of hematopoietic cell lineage markers that can reliably identify ASCs exclusively.

Over the past 6 years, many papers have reported on characterization of cell surface markers at stromal vascular fraction or at different stages of ASC culture. Considerable progress has been made towards phenotypic characterization of ASC; however, the expression profile changes as a function of time in passage and plastic adherence [61, 62]. Besides, some cell surface markers have been detected with highly consistent patterns of expression (CD13, CD29, CD73, CD90, CD105, CD166, CD133, CD45, CD31, MHC I, and MHC II) on the surface of ASCs by different literatures [16, 44, 47, 58, 63–65]. Still considerable heterogeneity, in the full range of ASC surface markers, had been reported. Some of these variations are found in the cell population within a single culture, indicating either the presence of mixed population of cells or the modulation of cell surface proteins during cell culture. Furthermore, the comparative analysis of phenotypic expressions had been confounded by the differences in thresholds used to report positivity in staining by different research groups [42, 49, 66].

To circumvent these barriers, minimal criteria with set of standards for identifying the MSC population from all sources have been proposed by the mesenchymal and tissue stem cell committee of the International Society for Cellular Therapy (ISCT) [67]. They reported that 95 % of the MSC population must express CD105 (known as endoglin and originally recognized by the Mab SH2), CD73 (known as ecto-5-nucleotidase and originally recognized by the Mab SH3 and SH4), and CD90 (also known as Thy-1), as measured by flow cytometry. Additionally, these cells must lack expression of CD45 (a pan-leukocyte marker), CD34 (primitive hematopoietic stem cells and endothelial cells), CD14 or CD11b (a monocyte/macrophage marker), CD79a or CD19 (markers of B cells), and HLA class II (expressed when stimulated by IFN γ). This standard has been accepted and followed for the identification of MSC by the scientific communities around the world.

Despite the in vitro identification of ASCs made possible through these aforesaid markers, their role with respect to other cell populations, such as side population, endothelial progenitor population, and specifically cell adhesion molecules within extracellular matrix, is not clear and there is a lack of consensus in the results. Although ASC shows very similar expression patterns as that of BMSC, it still remains unclear in the aspects of ASC specific marker [16, 44, 47, 58]. On the contrary, there are also reports specifying the variations exhibited among certain markers of ASC when compared to

BMSC and vice versa [14, 42, 47, 54, 63], further making cell surface marker expression study on ASC an arduous task. For example, it was identified that ASCs express CD49d and not CD106, whereas bone marrow MSCs express CD106 but not CD49d. This reciprocal expression pattern is interesting because CD106 is the cognate receptor of CD49d, and both these molecules represent part of a receptor–ligand pair that has an important role in hematopoietic stem cell homing to, and mobilization from, the bone marrow [68–71]. Despite the lack of CD106 in ASC, CD49d along with the higher expression of its counterpart, CD29, together forming VLA 4 is supposed to play a role in mobilization and homing [44, 47, 49, 54, 60, 72, 89]. In addition, it is predicted that high expression of CD44 is required for firm adhesion of MSC to endothelium. Data shows that CD44 is activated by PDGF [73] that plays an important role in exogenous migration to injured site by interaction with hyaluronate. Besides, CD13 was also identified to be a potent marker that plays a vital role in angiogenesis and migration. This wealth of knowledge on these markers about their crucial migration and homing evokes that these markers impersonate a CAM that performs these aforesaid functions in ASC. Although the existence and functionality of certain ASC specific markers are known, there is uncertainty among the specificity and functionality of several other markers of ASC.

Overall, the ASCs were identified by expressions of various markers such as STRO1, CD9, CD10, CD13, CD29, CD44, CD90, CD49a, CD49b, CD49d, CD49e, CD49f, CD54, CD55, CD59, CD166, CD71, CD117, CD19, CD146, and HLA-ABC. Similarly, there is evidence supporting that the surface markers CD31, CD45, HLADR, CD11b, CD14, and CD34 were decreased in expression or were lost with passage, suggesting that adherence to plastic and subsequent expansion will select for a relatively homogenous cell population compared with the SVF [18, 45, 47, 48, 54, 63, 65]. One of the key issues yet to be resolved is the absence of comprehensive information on certain specific markers of ASC, especially the cell adhesion molecule that interacts with the cytoskeleton of MSC. This might enhance the understanding of ASC as an instrument of curative therapeutics involved in the applications of neovascularization, angiogenesis, and treatment of other vascular disorders. Furthermore, these definitive cell surface markers of ASC would help not only to distinguish them from other cell populations in cell culture but also enable purification of ASC from uncultured SVF. As uncultured heterogenous SVF is been used in clinical trials extensively for treating a wide horizon of diseases, it is imperative to identify the heterogenous cell surface markers specific to SVF. The description on the existence of heterogenous and homogenous cell surface markers that can reliably specify ASC as depicted (Table 10.1) facilitates researchers to explore further therapeutic potentials of stem cells with SVF as well as ASC.

Table 10.1 Comprehensive analysis of cell surface markers in SVF and ASC

Markers	SVF	ASC	
Hematopoietic markers	CD34#	+	–
	CD14	ND	–
	CD45	ND	–
	CD117	ND	+
Mesenchymal markers	CD90	+	+
	CD73	+	+
	CD105	–	+
CAM molecules	CD49d#	+	+
	CD49a	+	+
	CD49b#	ND	+
	CD49e	+	+
	CD61#	ND	+
	CD62e#	ND	+
	CD63#	+	+
	CD54#	+	+
	CD50	ND	–
	CD51	ND	+
	CD56	ND	–
	CD55	ND	+
	CD29	+	+
	CD11a	ND	–
	CD11b	ND	–
	CD11c	ND	–
	CD44	+	ND
	CD31	+	–
	CD9	ND	+
	CD106	+	–
	CD166	+	+
	CD104	ND	–
	CD144	–	ND
	CD146#	+	+
Surface enzyme	CD13	+	+
	CD10	ND	+
	ALDH	+	+
Growth factors	CD140b	ND	+
	VEGFR-2	–	–
Histocompatibility antigens	HLA-ABC	ND	+
	HLA-DP	–	–
	HLA-DQ	–	–
	HLA-DR	–	–
Side population	ABGC2	+	+
Cytoskeleton marker	α-SMA, vimentin	ND	+
Complement cascade	CD55	ND	+
	CD59	ND	+
Receptor molecules	CD71	ND	+
Endothelial marker	von Willebrand	–	–
Pluripotent markers	Oct-4	ND	+
	NANOG	ND	+
	SOX2	ND	+

Refs. [14, 42, 44–47, 52–54, 63, 65, 75, 92, 152, 186–193, 208]
+ expressed, – not expressed, # variable, *ND* not done

Differentiation Potency

Another property put forward by ISCT that potentially identifies MSC is by its differentiation potency. The cells must be able to differentiate to mesodermal lineages of osteoblasts and adipocytes to demonstrate bone and fat phenotypes, respectively, under standard in vitro differentiating conditions [67]. To investigate multipotency, several researchers had demonstrated the multilineage differentiation ability of subcutaneous adipose-derived stem cells [15, 16, 42, 74, 75]. For instance, Rodriguez and his co-workers [76] created a single clone from fast-adherent ASCs and proved that 2 out of 12 clones were able to undergo multilineage differentiation [48]. The remaining ten clones had bipotent capacity. These findings indicate that a high percentage of ASCs have multipotential and pluripotential capacity in vitro to differentiate into the major mesodermal and ectodermal lineages. Adipose tissue-derived mesenchymal stem cells naturally differentiate into mature adipocytes [15, 16, 42, 74, 75]. Upon treatment with adipogenic induction medium containing 3-isolbutyl-1- methylxanthine (IBMX), dexamethasone (DEX), indomethacin and insulin, ASCs were found to develop intracellular lipid vacuoles which coalesce and give rise to a single, cytoplasm filling vacuole. Besides, the definite markers of adipogenesis [76], ASCs also express a wide variety of metabolic markers such as glycerol-3- phosphate dehydrogenase (GPDH), lipoprotein lipase, peroxisome proliferator-activated receptor γ(PPARγ), leptin, adipocyte fatty acid-binding protein (Ap2)11, CCAAT/enhancer-binding protein (C/EBP), and glucose transporter 4 (Glut4).

Similarly, ASC differentiation towards the osteogenic cell lineage is well established for in vitro and for in vivo animal tissue engineering models [15, 16, 42, 74, 75, 77]. A clinical observation is in part responsible for the discovery of the osteogenic differentiation capacity of ASCs. A rare disorder named "progressive osseous heteroplasia" together with the capacity of MSCs to convert into the osteogenic lineage led to the assumption that ASCs are likewise able to differentiate into osteocytes [78]. Osteogenic induction of ASCs can be achieved by similar culture conditions as used in MSCs, including supplementation with ascorbic acid together with 1-alpha,25-dihydroxyvitamin D3 (1,25(OH)2D3), the hormonal metabolite of vitamin D, or dexamethasone [79]. Under osteogenic differentiation medium, ASCs are capable of expressing diverse genes and proteins found in the osteoblast's phenotype: type I collagen, alkaline phosphatase, osteocalcin, osteonectin, osteopontin, parathyroid hormone (PTH) receptor, bone morphogenetic protein-2 (BMP-2), BMP-4, BMP receptors I and II, bone sialoprotein, and RunX-1 [18, 79, 80].

Earlier, mesenchymal stem cells were considered as capable of only forming tissues of mesodermal origin, as has been demonstrated above. However, in recent years this phenomenon has been challenged by several reports, and it is no longer surprising that mesenchymal stem cells, having originated in the mesoderm, can cross the germ layer boundary and undergo different mesenchymal lineage conversions of ectodermal and endodermal origins. This process has been termed as transdifferentiation or plasticity. This is evident from the clonal expansion of adipose-derived stem cells which proved that at least one part of the plasticity is situated in a fraction of multipotent cells [45]. This concept that mesenchymal stem cells possess a far wider potential of transdifferentiation is also supported by literatures [81]. The perception of the far wider potential of multidifferentiation potency of ASCs as depicted (Table 10.2) has led to considerable excitement with regard to its potential therapeutic applications in regenerative medicine and tissue engineering [15, 59]. Nevertheless, further needs to be explored on the differentiation potency of ASCs in order to achieve curative therapeutics for all diseases.

Prerequisite for Ex Vivo Expansion

Adipose tissue-derived stem cells were identified to possess the key characteristics of MSCs as reported by ISCT, including the ability to form plastic-adherent fibroblastic morphology, extensive proliferative capacity, the ability to express several common cell surface antigens, and the ability to differentiate into several mesodermal lineages, including the bone, fat, and cartilage. Hence, ASCs are recognized an attractive and abundant stem cell source in recent years, with therapeutic applicability in diverse fields of tissue repair and regeneration. To take this initiative to the next step, on the front line, it is important to isolate and generate a large clinical quantity of MSCs, while retaining stem cell characteristics, immunosuppressive capacity, and multilineage differentiation potential. Reaching the estimated clinical dose of cells per kilogram body weight of adult patients is highly challenging. Hence, in vitro expansion of MSC is a prerequisite to obtain clinical dose for subsequent therapeutic applications using ASCs.

The use of MSCs for clinical approaches in many fields of medicine first requires that the biosafety of these cells be carefully investigated through appropriate and sensitive tests. Indeed, documentation on the absence of transformation potential in cultured MSC before infusion of these cells into patients is of utmost importance. Furthermore, as MSC possesses a greater propensity for ex vivo expansion, ISCT reported that extensively passaged cells may be well served by verifying a normal karyotype to reduce the probability of chromosomal abnormalities, including potentially

Table 10.2 Induction of multilineage differentiation of ASCs in vitro

Germ layer	Induction factors	Genes upregulated
Mesoderm		
Adipocytes	3-Isobutyl-1-methylxanthine (IBMX) + dexamethasone(DEX) + indomethacin + insulin [18, 207]; ascorbic acid + insulin + sodium selenite + triiodothyronine + IBMX + DEX + rosiglitazone [48]; IBMX + DEX + insulin + pantothenate + biotin + rosiglitazone [45]	PPARγ, CCAAT/enhancer-binding protein α (C/EBP α), lipoprotein lipase (LPL), adipocyte fatty acid-binding protein (FABP4/aP2), leptin
Osteoblast	DEX + ascorbate-2-phosphate + β-glycerophosphate [18, 207, 208];1,25$(OH)_2D_3$ + β-glycerophosphate + ascorbic acid + BMP-2 + DEX + valproic acid [77, 194–196, 209, 210]; ascorbate-2-phosphate + β-glycerophosphate + BMP-2 [43]	Type I collagen, alkaline phosphatase, osteocalcin, osteonectin, osteopontin, PTH, BMP-2, BMP-4, BMP receptors I and II, RunX-1, Bone sialoprotein, ALP
Chondrocytes	Insulin + TGF- β + ascorbate-2-phosphate [18, 197–199, 201, 211]; DEX + ascorbate-2-phosphate + sodium pyruvate + TGFβ1 + ITS premix [43], TGFβ3 + BMP-6 + DEX + ascorbate-2-phosphate + proline + pyruvate + ITS premix [208]	COLL II, COLL VI, aggrecan
Cardiomyocytes	5-azacytidine [206] Transferrin, IL-3, IL-6, VEGF	Connexin-43
Myogenesis	Hydrocortisone + DEX [18]	Desmi, MyoD, dystrophin, telethonin
Endoderm		
Beta cells	Glucose + nicotinamide + activin-A2 + exendin-4 + HGF + pentagastrin + B27 + N2 [81]	Isl-1,Pax-4,Pax-6, PDX-1, Ngn-3, insulin, nestin, glucagon, somatostatin, NeuroD, Nkx2.2, Glut2, Ipf1
Hepatocytes	HGF + bFGF + nicotinamide + DEX + insulin + OMS + transferrin + selenious acid + linoleic acid + BSA + ITS [204] DMSO + rhHGF + rhOSM [168]	Albumin, α-fetoprotein, enhancer-binding protein beta C/EBPβ, albumin (ALB), transthyretin (TTR), cytochrome 2E1 (CYP 2E1)
Ectoderm		
Neurons	β-Mercaptoethanol [42]; β-mercaptoethanol + trans-retinoic acid + PDGF + bFGF + GGF-2 [205]; butylated hydroxyanisole + valproic acid + forskolin + hydrocortisone + insulin + KCl [202];	
5-azacytidine + NGF/BDNF/bFGF + B27 [164]	MAP2, γ-enolase, NeuN, intermediate filament m, nestin, glial fibrillary acidic protein (GFAP), β-III tubulin, oligodendrocyte marker O2, glutamate receptor subunit NRN 1 and NRN 2	

transforming events [67]. Considering these facts, there have been continuous attempts in optimizing culture conditions for ASCs that retain stem cell characteristics that could be beneficial for clinical and therapeutic applications [46, 82–84].

Despite the wide prevalence of mesenchymal stem cells from adipose tissue and their observed benefits to their use in both in vitro and in vivo models of certain human diseases [15, 16, 42, 74, 75, 77], clinical trials using ASC have not reached major success. Research exists on overcoming the barriers to maximize the beneficial effects of ASC for their use in treating wide horizon of diseases. One such anticipated barrier might be due to failure of the transplanted cells to survive, proliferate in numbers, and differentiate into tissue-specific cells, which could then incorporate into diseased tissue, repopulate the area with healthy tissue, and thereby exerting an appreciable effect of improvement. Although the reason for the same is not fully understood, it is hypothesized that MSC might enter senescence and start losing their characteristics soon after infusion [85]. Hence, optimization of culture condition and maintenance of ASC characteristics under extensive culturing in vitro are crucial to resolve this issue.

Furthermore, it was identified that ASCs have profound immunomodulatory effects in vivo and are regarded as hypoimmunogenic cells [65], and hence ASCs from allogenic donors might constitute a valuable alternative source of stem cells for their use in case of transplantation. Thus, ASC can be a promising tool that forms the basis for new strategies in immunoregulatory cell therapy and allogenic cell transplantation in regenerative medicine. In order to achieve this, ASCs have to be expanded extensively to yield cells that retain its characteristics substantially enough to give appreciable effect to cure diseases. By achieving this attribute, a large quantity of allogenic donor-derived MSCs can be obtained from subcutaneous adipose tissue in a less time-

consuming, noninvasive, cost-effective, efficient, and safe source of clinical cell transplantation.

In accordance to these, several reports have focused on studying the efficacy of retention capacity of its characteristics at prolonged culture condition of both rat and human adipose tissue-derived mesenchymal stem cells. Both murine and human stem cells were found to retain their properties evidence for the possibility of their characteristics until prolonged culturing. They were able to preserve their long-term stem cell characteristics and differentiation potential even at longer passages [46, 82, 83]. The human subcutaneous adipose tissue showed high telomerase activity that could be maintained for more than 100 population doublings. The karyotype was found to be normal and it was positive for certain pluripotent markers retaining its efficacy until later passages. Thus, evidence is available proving that subcutaneous adipose tissues possess properties of true stem cells, which were retained even after extended in vitro culturing, thereby rewarding a prerequisite for possible successful cell-based therapies. However, on the contrary, ASC under extensive expansion condition was reported to lose its characteristics of retention and differentiation ability [84, 86, 87]. In addition, another group observed spontaneous malignant transformation of adipose-derived hMSC in vitro [88], thus creating a perplexity and hindering the progress of ASC towards therapeutic interventions.

Hence, enhanced research work on ASC regarding maintenance of its quintessential properties in early and later passages without genetic or epigenetic changes before its use in clinical transplantation is of utmost importance. In accordance to this, our laboratory had reported recently that ASCs in early passage condition obtained from various donors (difference in age, gender, and BMI) showed a consistency related to its expression of cell surface markers, growth potency, and differentiation ability [89]. This study, along with the reports of immunomodulatory properties of ASCs explained earlier [65], thus, demonstrate the importance of cryopreservation of cultured ASCs. This identification puts forth a breakthrough challenge of using these cultured ASCs towards clinical applications that can be subsequently used for effective tissue repair and regeneration in a noninvasive manner. However, in order to achieve this challenge, our group investigated whether subcutaneous adipose-derived mesenchymal stem cell could be retained in extensive culturing without losing their property. The hypothesis was achieved by culturing the ASC in five different media, viz., DMEM LG, DMEM HG, Alpha MEM, DMEM F 12, and DMEM KO, as media used for cell culture have a significant impact on growth and differentiation of ASC. We proceeded a step forward to demystify a number of myths of ASC. In our study, optimization of culture media was successfully achieved by extensively propagating mesenchymal stem cells from subcutaneous fat until passage 25. The study revealed the possible use of ASCs from subcutaneous fat in curative therapeutics by demonstrating retention characteristics of MSC for more than 6 months in all media used, which was confirmed by various attributes in view of its expression profile, proliferation, differentiation, and normal karyotype in its extensive culturing condition. Furthermore, *optimization of basal media appropriate for culturing ASCs from subcutaneous fat based on its growth curve and population doubling time was also identified, thereby permitting the scientist to choose their pertinent media according to their type of research and therapeutic implications* [90].

Yet another obstacle that can potentially hinder the application of cultured ASCs for its use in cell-based therapies is the fetal bovine serum (FBS), as culturing cells aimed for clinical therapy in FBS is an unsuitable option with respect to patient safety [91]. Although from cell culture point of view FBS provides the cells with essential nutrients and growth factors [92], species of origin and serum concentrations affect the proliferation of ASCs [93–95]. Human cells exposed to xenogeneic (i.e., animal derived) products originating from cell culture reagents may transfer xenogeneic antibodies, such as Neu5GC, into the human body upon transplantation, raising the risk of triggering a severe immune response in the recipient [96–98]. Several researchers have thus focused on identification of FBS replacement with human serum derivatives to support equal or higher proliferation rates and multilineage differentiation capacity of ASCs [93, 95, 99, 100]. AutoHS (autologous human serum) is perhaps the obvious option for clinical applications, since it eliminates the problem of introducing xenogeneic or allogenic antibodies into the patient. However, there are conflicting results of the superiority of autologous human serum compared to FBS in terms of proliferation rate and differentiation potential [101]. Oreffo et al. [102] reported improved osteogenic and adipogenic differentiation using autoHS compared to FBS, and Yamamoto et al. [103] showed similar results for osteogenic differentiation using autoHS versus FBS. However, utilizing autoHS for large-scale stem cell production for clinical applications is impeded by limited availability and high variability in cell growth in autoHS [92, 101, 104, 105]. Development of completely defined, SF/xeno-free (XF) medium compositions for expansion of adult stem cells is still in its infancy with only few papers published, however, with highly promising results [106–108]. Importantly, the culturing formula must also be capable of expanding the cells multifold in a minimum number of passages, since long-term in vitro culture may be time consuming and at times might alter the biology of ASC [109, 110]. Further research pursuit in this aspect might create a hope in the light of existing perplexity to bring subcutaneous fat as a frontline source of both autologous and allogenic therapeutic stem cells in the field of regenerative medicine.

Applications in Regenerative Medicine

Current treatments for various degenerative disorders rely on surgical interventions and drugs that modulate the system, but these have their own limitations when it comes to regeneration of damaged tissues and cells. To address this shortcoming, cell-based therapies are gaining importance. Regenerative medicine is a multidisciplinary field of research that is rapidly expanding with the advances in the technologies. It involves the use of a stem cell, growth factor, and a biomaterial that restores function by enabling the body to repair, replace, and regenerate or rejuvenate damaged, aging, or diseased cells, tissues, and organs. Adult stem cell research has been hailed for its potential to revolutionize the future of regenerative medicine with the ability to regenerate damaged and diseased organs. Detailed study on adult stem cells in the past decade has kindled useful knowledge about developmental, morphological, and physiological processes that form the basis of tissue and organ formation, maintenance, repair, and regeneration.

Conceptually, and from a practical standpoint, the bone marrow has always been considered a rich source of stem cells for regenerative medicine. It is known to possess heterogenous stem cells that participate in all steps of hematopoiesis and tissue regeneration. Despite its colossal potential and benefits in certain diseases, stem cells derived from the bone marrow have not been promising to attempt curative therapeutics for all diseases. Hence, adipose tissue-derived stem cells flourished as an alternative source to play a potential pivotal role in regenerative therapeutics. Subcutaneous adipose tissue is an abundant, accessible, and replenishable source of both uncultured/heterogenous stromal vascular fraction cells and cultured/relatively homogenous adipose-derived stem cells. The basic, experimental, and clinical research on SVF/ASC has expanded exponentially over the past decade. Cell-based therapy using ASCs presents a unique opportunity for their use in tissue repair and regeneration. The important experimental findings using SVF/ASC in recent years in treating wide range of diseases are detailed below, thereby laying a blueprint for ASC in cellular replacement and regenerative medicine.

Mesodermal Applications

Bone Diseases

Bone diseases, such as osteopenia and osteoporosis, affect several millions of patients throughout the world. Ongoing studies for repairing bone defects are performed in pharmacology, gene, and cell therapies. Suitable cells needed for tissue engineering should exhibit immunocompatibility and self-regenerative potential. Osteoblast differentiation represents a crucial event during skeletal tissue formation, bone repair, and bone remodeling [111]. The in vitro osteogenic potential of ASCs has been documented in multiple studies [18, 42, 74, 79, 80, 112, 113] as described above. Furthermore, these cells have also proven to regenerate bone defects in the in vivo animal and human models as elucidated below. In efforts to utilize this potential for tissue-engineered bone repairs, many laboratories have begun seeding osteogenically differentiated ASCs onto various scaffolds and biomaterials. The biomaterials that rendered the most significant result are PGA [74], atelocollagen [112], and hydroxyapatite/tricalcium phosphate (HA-TCP) [114]. The first use of autologous ASC for osseous repair has been reported in the treatment of craniofacial defects, for a bilateral calvarial defect in a 7-year-old girl [115]. Due to the restricted amount of BM available in patients with calvarial defects, the trial followed a complex clinical course characterized by the application of ASC to the defect in a single procedure together with autologous iliac crest bone and fibrin glue combined with resorbable sheets. New bone formation as well as nearly complete calvarial continuity was evident 3 months after the reconstruction. It is believed that strong paracrine signaling from the underlying dura mater and the osteoinductive properties of the apatite coating may have played a role in these divergent outcomes. Further successes have been observed in a rat cleft palate model. Osteogenically differentiated ASCs seeded onto polylactic acid scaffolds led to near-complete palatal repair at 12 weeks when implanted into the defect. In comparison, no bone formation was seen with acellular scaffolds and those seeded with undifferentiated cells [116]. Furthermore, the author harvested autologous fat tissue from a 65-year-old male who had undergone a hemimaxillectomy 28 months earlier due to a large recurrent keratocyst, expanded the cells in culture, mixed them with BMP-2, and seeded them onto a beta-tricalcium phosphate scaffold formed into the shape of the defect. Eight months after this construct was implanted into the patient's rectus abdominis muscle, the construct was resected and transplanted into the maxillofacial defect. The patient regained full oral function. Although evidence to date suggests that ASCs may one day be useful in the treatment of difficult osseous repairs, further investigations are needed to determine their ultimate safety and efficacy in the clinic.

Soft Tissue Defects

One of the most intuitive uses of ASCs is for the replacement of adipose tissue itself. Large soft tissue defects are a common problem following trauma, burns, and oncological resections, such as mastectomy. In addition, thousands of patients annually require cosmetic treatments to smooth wrinkles, fill in cheeks, and otherwise augment natural subcutaneous adipose compartments. The material currently used in soft tissue regeneration, which includes collagen, hyaluronic acid,

silicon, and other filler materials, has several disadvantages such as high cost, immunogenicity, and allergenicity and the risk of transmitting infectious diseases. Although autologous fat grafts are available, their one limitation is the poor long-term graft retention in current clinical practice [117]. The transplanted graft can lose volume over time due to tissue resorption that can result in the loss of the original graft volume [118]. In order to develop more physiological alternatives for soft tissue reconstruction, several laboratories have investigated the possibility of creating tissue-engineered cell-seeded scaffolds for the generation of de novo adipose tissue. The ideal solution for soft tissue regeneration would promote the regeneration of vascularized adipose tissue to completely fill the defect volume. Min et al. demonstrated in an in vivo murine model that the transplantation of fat tissue with non-cultured ASC improved long-term graft retention and a higher density of capillaries 6 and 9 months after transplantation [117]. The reasons for these successful results might be the proangiogenic growth factors secreted by ASCs. Initial success rate has been seen in animal models injected with cells seeded with artificial scaffold [119–121]. In each trial, cell-seeded grafts showed significant neovascularization of the implant, as well as penetration of the preadipocytes or ASCs into the scaffolding, and their differentiation into mature lipid-laden adipocytes. However, the influence of variables such as porosity, biomaterial composition, and seeding density has been under continuous investigations for the optimization of the constructs to improve adipogenesis.

Yoshimura and co-workers used adipose-derived SVF cells for soft tissue augmentation by a novel strategy called cell-assisted lipotransfer (CAL) for treatment of facial lipoatrophy and for breast augmentation. It was identified that ASC supplementation has improved its efficacy and improved facial contour, with no adverse effects, although there is no statistically significant difference identified. Furthermore, breast tissue augmentation and reconstruction trial had been reported by Yoshimura and colleague [122, 123]. The SVF was isolated from half of the aspirated fat tissue, recombined with the remaining half, and used in combination with lipoinjection in over 50 patients. These results showed no evidence of fibrosis or adhesions and improved fat grafting by the SVF cells with the retention of volume for 12 months. Developing strategies to reconstruct larger tissue defects, however, remains a formidable challenge due to the inherent ischemic conditions within larger transplants and the time necessary for the establishment of an extensive vascular network.

Cartilage Defects

Clinical cartilage repair has remained an elusive goal for some time. Autologous [124] and allogenic [125] chondrocyte transplants have been used successfully, but are limited by donor site morbidity and the slow repairs seen, respectively, with these approaches. Recognition of the chondrogenic differentiation potential seen in many stem cells has led to the exploration of an alternative source of cells. Chondrogenic potential, described in vitro in ASCs, includes evidence of cell condensation into nodules and the production of an extracellular matrix rich in proteoglycans and collagen type II [126–131]. It has been proved successful for a minimum period of 12 weeks when implanted with alginate constructs subcutaneously in nude mice [129]. A direct comparison of the in vitro chondrogenic potential of ASCs and BMSCs examined similarities in histological staining and gene expression [131]. In vivo experiments using ASC spheroids had been identified and were successful at generating cartilage-like tissue [132]. Induced spheroids were implanted between two muscle bellies in immunodeficient mice. At 6 weeks, the implants were harvested and found to have produced a cartilage-like tissue consisting of cells within lacunae surrounded by a gel-like extracellular matrix in the absence of any fibrous network. Although this study demonstrates an in vivo potential for differentiation intramuscularly, no physiological models of cartilage repair have yet been tested. The cartilage, particularly articular, primarily serves a structural and mechanical function in the body. To this end, one laboratory is investigating what effects scaffold material [133, 134], oxygen tension [133], and media composition [133] have on the biomechanical properties of ASC-seeded constructs. Clear differences are seen depending on the combination of factors used; however, nothing yet approaches the mechanical properties of mature cartilage. Thus far, results suggest that future in vivo models may demonstrate a potential for ASCs to enhance the healing of debilitating osteochondral diseases. Repairs with the resilience necessary for weight-bearing joints, however, will probably be more difficult to develop. It remains controversial whether ASC cells are perfect for cartilage engineering. However, ASC seems to have a greater chondropotential effect and further enhanced work on the same might improve engineering of cartilage in vitro as well in vivo.

Cardiac Repair and Neovascularization

Congestive heart failure, a common clinical condition that results from acute ischemic events and diffuse progressive weakness, is essentially a failure of the myocardium. Existing pharmaceutical drugs in the market can temporarily resolve the effect, but later drive the heart harder and might further weaken the muscle and play a little role to repair damaged tissue. The use of stem cells to regenerate damaged heart tissue is advocated as the new treatment for heart failure secondary to heart disease or severe myocardial infarction. Several recent reports suggest that ASCs may emerge as a

promising source for cardiac therapeutics. In vitro differentiation, including morphological changes, spontaneously beating foci, and the expression of cardiac-specific markers, has been demonstrated by multiple laboratories [135–137].

The injection of both cultured and freshly isolated ASCs into the peri-infarct area of the heart of mice has been identified to potentially improve cardiac function in experimentally induced myocardial injury. Two functional studies using in vivo infarction models were recently presented at the 2005 meeting of the International Fat Applied Technology Society (IFATS). In addition, cellular cardiomyoplasty appears hopeful in light of recent reports that mesenchymal stem cells produce cardioprotective factors in sufficient amounts to improve myocardial function within 72 h after cell transplantation [138]. This effect is confirmed by animal studies and clinical studies that treatment group has increased capillary densities [139, 140]. However, as smooth muscle differentiation, the in vivo differentiation of ASCs into cardiomyocytes is still controversial. Strem et al. reported that ASCs express cardiac markers in vivo 2 weeks after injection, but Cai and his co-workers [141] found that intramyocardially injected ASCs differentiated into smooth muscle cells but not into cardiomyocytes in rats.

The therapeutic potential of ASCs may be further enhanced through their effects on neovascularization. ASCs in culture are known to secrete proangiogenic factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor, and transforming growth factor-β [142]. Under hypoxic conditions, they have shown enhanced secretion of VEGF and of an unidentified antiapoptotic factor [142]. In vitro, endothelial differentiation of ASCs has likewise been described in Matrigel cultures. In addition to the characteristic tubelike structures, differentiated ASCs also expressed endothelial markers, such as CD31, Flk-1+, and von Willebrand factor [143, 144]. Earlier studies have demonstrated that the beneficial effects are mediated by angiogenic and antiapoptotic cytokines produced by mesenchymal stem cells [138]. In vivo results from a hind limb ischemia model demonstrated increased capillary density and perfusion in limbs treated with ASCs, further supporting the cells' angiogenic potential [142, 145]. These promising results may enhance the future application of ASCs in myocardial infarction, other cardiovascular diseases, and neovascular diseases that are one of the leading causes for morbidity and mortality in Western countries.

Muscular Disorders

Muscular dystrophies are a clinically and genetically heterogenous group of disorders characterized by progressive degeneration and loss of skeletal muscles [146]. Adult skeletal muscle has the potential to regenerate new muscle fibers by activating a population of mononucleated precursors, which otherwise remain in a quiescent and nonproliferative state [147]. However, the continuous and gradual muscle degeneration in progressive muscular dystrophies leads to depletion of satellite cells, and consequently, the capacity to restore the skeletal muscle is lost [148, 149]. Duchenne muscular dystrophy, an X-linked lethal disorder that affects 1 in 3–4,000 male births, is the most prevalent form of muscular dystrophy [150]. In case of muscle injury, muscle satellite cells are activated to become myogenic precursor cells. These cells divide and fuse to repair the damaged muscle. However, the mature muscle satellite cells represent 1–5 % of the total muscle cells and their potential for self-renewal decreases with age [151]. In DMD, the intense degeneration that occurs in muscle fiber exhausts the ability of satellite cells to proliferate and replace damaged fibers [149].

Initial efforts using myoblast transfer demonstrated short-term benefits, but were ultimately limited by poor cell survival, immune rejection, and poor migration of transplanted cells. There evolved the strategy to treat muscular diseases by restoring dystrophin levels in patients with DMD using cellular therapies. Those cell-based therapies that may replenish the exhausted supply of satellite cells may be particularly suited to prevent this decline. Several groups demonstrated that ASC is capable of differentiating along multiple lineages, including myocytes, in presence of lineage-specific induction medium [152]. This was demonstrated by the presence of certain characteristic markers and the formation of multinucleated myotubules [152–154]. Two hypotheses exist on the contribution of ASC to muscle regeneration: de novo generation of muscle-specific cells from ASC and modification in gene expression after direct fusion of ASC with host cells.

In the first in vivo study reported by F Bacou [155], ASCs were injected into the anterior tibialis muscle of rabbits following cardiotoxin-induced injury. Although this study was statistically significant, clinically noticeable improvements were uncertain. Other in vivo studies showed that implantation of ASC in mdx mice restored dystrophin expression in the dystrophic mouse cells [48]. Allogenic ASCs injected intravenously or directly into the affected muscle could restore muscle function in a murine muscular dystrophy model without any signs of immune rejection [156]. Another study, conducted by Kim and his co-workers [100], used PLGA spheres attached to myogenically induced ASCs to inject subcutaneously into athymic nude mice. Injected ASCs differentiated into muscle cells and regenerated new muscular tissue. Although the demonstration that ASCs have myogenic potential both in vitro and in vivo is promising, it is still unclear whether ASCs directly differentiate into myogenic lineage cells or they become incorporated into muscle fibers via cell fusion [203]. However, further work is needed to establish the methods necessary to treat progressive diseases.

Ectodermal Applications

Neurodegeneration

Neural tissue has long been regarded as incapable of regeneration, and the identification of stem cell population capable of neuronal differentiation has generated intense interest [157, 158] amidst the scientific community. The ability to differentiate into neuronal lineage is yet another property of ASC that provides evidence for its range of plasticity, much broader than originally thought. The ASC can achieve neurosphere formation when cultured at high density. Subsequent culture of the spheroid bodies on the laminin leads to final neural differentiation. Differentiated cells can be identified with the protrusion of extensive cell processes with a neuronal morphology consistent with a neural phenotype and expression of early neural precursors. Further trials have successfully induced the expression of an even broader range of mature neuronal and glial markers [159–162]. It remained as a controversy as to whether such neurons are electrically active and functional with all essential characteristics of mature CNS neurons. However, several in vivo studies carried out in animal model and humans, so far, had given maximum promising results for the treatment of cerebral ischemia, spinal cord injury, as well as neurological diseases [163]. ASC has also been identified to migrate into the injured cerebral lesions after ischemic stroke and has improved functional deficits and motor recovery. Despite major improvements, it is uncertain whether transplanted cells contribute to the direct replacement of lost neurons or provide a supportive role for existing in situ stem cells and injured neurons. In a coculture model, Kang and his coworkers [164] studied the interactions between neural stem cells and adipose-derived stem cells. In comparison to laminin-coated dishes, ASC feeder layers showed an ability to support the differentiation and survival of neural stem cells over 14 days in culture. The functional improvements seen after stem cell infusion in vivo may be a result of this supportive ability. Engraftment studies have demonstrated that ASCs cross the blood–brain barrier [155], but their role after they arrive is unclear. Although certain aspects of neural differentiation are unclear, it is important to note that evidence exists for functional improvements in ischemia, traumatic brain injury, as well as spinal cord injury using ASCs. However, further demonstrations on ASCs are required to reasonably prove the fact beyond doubt and to advance its applicability.

Endodermal Application

Liver Cirrhosis and Hepatic Failure

Most liver diseases lead to hepatocyte dysfunction with the possibility of eventual organ failure. Liver transplant may be the only option to treat patients with heavily damaged livers in case of both acute and chronic failures. The major barrier for this transplantation is the shortage of donors and immune rejection, which is still unsolved. Cell-based therapies have been under investigation for the past decade to fill this void of failure of treatment of hepatic dysfunction. The replacement of diseased hepatocytes by stem cells and the stimulation of endogenous or exogenous regeneration by stem cells are the main aims of liver-directed cell therapy. There is growing evidence of reports describing the hepatocytic differentiation potential of ASC [165–168] and the effective functions of these differentiated hepatocyte-like cells not only in vitro but also in vivo. In 2005, Seo and his co-workers reported that hASCs cultured in media supplemented with growth factors and cytokines yielded a cell population that expressed a number of hepatocyte-specific functions, such as albumin production and urea synthesis. A subsequent in vivo model tracked human ASCs injected into mice 2 days following exposure to a known hepatotoxin. Donor cells engrafted in the liver assumed a hepatocyte-like morphology and began expressing albumin [167]. Similarly, transplantation of hASCs into SCID mice with acute liver failure caused by CCl4 injection revealed that undifferentiated hASCs were able to engraft into the liver and improve its function [168]. Although these data suggest that transplanted cell may differentiate in vivo into mature hepatocytes, they did not address the rate of recovery of liver function, and the approach of using hASC is at its infancy. Plenty of issues still remain to be investigated before application is justified in clinical setting for enhanced tissue repair and recovery of organ failure.

Diabetes Mellitus

Diabetes is a major malady that causes a large portion of epidemic deaths worldwide. The epidemic proliferation of diabetes is at such a high rate that new drugs and other therapeutic approaches are required to curb it. Over the last few decades, the main therapeutic approach to insulin-dependent diabetes has confined to the use of insulin injections [169], but was proven ineffective in replacing normoglycemic level. As an alternative strategy, insulin gene therapy focuses on converting non-β-cells into insulin-producing cells by introducing insulin synthetic genes with secretary techniques [170, 171]. Transplantation of islets of Langerhans has been shown to be successful in experienced centers, but this therapy can be offered only to a very limited number of patients due to shortage of donors and immune rejection [172–176]. Regeneration of pancreatic β-cell has emerged as a recent advancement in stem cell technology. Insulin-secreting cells generated from stem cells could represent an attractive alternative.

Transplantation of pancreatic beta islet cells had showed that approximately 70 % of the patients treated for type I

diabetes achieved insulin independence [177]. Autologous islet transplantation (AIT) has proved to be an efficacious treatment strategy to prevent surgical diabetes. AIT has also proven efficacious in normalizing glycemic levels in patients with benign pancreatic tumors and pancreatic trauma [178]. AIT has been shown to be beneficial for diabetes treatment; the transplanted islets do not suffer allogenic rejection and diabetogenic antirejection drugs are not required. However, the limited availability of autologous pancreatic cells limits the applicability of this technique. Considering these prevailing conditions, autologous stem cells as well as autologous stem-/progenitor-derived insulin-secreting islet-like clusters are gaining importance. Transdifferentiation of subcutaneous adipose tissue-derived stem cells into beta cells has been carried out in vitro [81] using multistep differentiation procedure, providing direct evidence that human ASCs could be programmed to become functional insulin-producing cells. These generated islet-like clusters were confirmed to secrete insulin in response to glucose stimulation and express various molecules that resembled those expressed by pancreatic beta cells, such as Isl-1, Pax4, Pax6, pancreatic/duodenal homeobox 1 (Pdx1), prohormone convertase (PC) 1/3, PC2, Kir6.2, glucose transporter (Glut) 2, glucokinase (GK), as well as the islet gene insulin. As the numbers of diabetic patients are increasing in recent years, it is of utmost importance to focus more on stem cell-based therapy for diabetes treatment. In lieu of this, a few but exciting reports from clinical trials conducted at different parts of the world are highlighted. Adipose-derived stem cells have been used as a novel therapy for patients suffering type II diabetes, where autologous activated adipose-derived stem cells were given as intravenous or direct catheter injection into the pancreatic artery of the patients proving its efficacy and usefulness in diabetes treatment (NCT01453751). There have also been studies confirming the efficacy of intravenous administration of stromal vascular fraction as a treatment of type II diabetes (NCT00703612). These data have proven adipose tissue as a safe, efficient enormous source for stem cells for its use in diabetes treatment. However, further research into the factors and in vivo mechanisms of pancreatic development will augment ASC application in curative diabetes and diabetes-related disease treatment.

Other Applications

Crohn's Disease

Crohn's disease, a chronic bowel disease characterized by bloody stools, diarrhea, weight loss, and autoimmune-related symptoms, usually affects young people between the ages of 18 and 40 years. Conventional treatment using corticosteroids, immunosuppressant, and biological drugs for inflammatory complications with surgery or stenosis (narrowing of the intestinal lumen) or fistulas (openings from the intestinal lumen to other organs, such as the intestine, bladder, vagina, or skin) led to deteriorated quality of life. Therefore, treatment of this disease using innovative cellular therapy was a need and is promising as shown by a number of successful clinical trials [179, 200]. In a phase I trial with patients with fistulas unresponsive to standard treatment, cultured ASCs were directly injected into rectal mucosa, and 75 % of cases healed completely. In a phase IIb trial, the proportion of patients who achieved fistula healing was significantly higher with ASCs than with fibrin glue (Table 10.3).

Wound Healing

Several studies have evaluated the potential therapeutic effects of ASCs on wound healing. Local implantation of ASCs has been found to be effective in supporting epidermal healing in full-thickness skin wounds of pigs, as well as in rats, in which the survival area of ischemic skin flaps was significantly increased by local injection of autologous ASCs. Rigotti and his co-workers [180] reported successful results after injection of lipoaspirates containing ASCs to wounds caused by postmastectomy irradiation. According to ultrastructural analysis, the early stages of tissue mesenchymalization were observed after application of lipoaspirates, and a tissue resembling normal mature adipose tissue was formed at the site of application. The authors commented that the effect of lipoaspirate on wound healing is largely due to the angiogenic growth factors secreted by ASCs [181]. These results were valuable in terms of showing the safety and feasibility of ASCs for clinical wound management.

GVHD

Graft-versus-host disease (GVHD) constitutes the most frequent complication associated with the transplantation of allogenic cells. SVF and ASC both serve as an effective treatment for steroid-refractory acute graft-versus-host disease after allogenic transplantation owing to immunosuppressive property of MSC. It is being revealed from the immunosuppressive effect of MSCs in vitro and in preclinical animal models that MSCs may be used for the prevention and treatment of graft-versus-host disease (GVHD), in organ transplantation to prevent rejection, and in autoimmune disorders. Yanez and co-workers reported that adipose-derived stem cells do not generate in vitro alloreactivity of incompatible lymphocytes and suppressed the lymphocyte proliferative response to mitogens and alloantigens [182]. The study also demonstrates that MSCs obtained from third-party donors were well tolerated and exerted in vitro and in vivo

Table 10.3 An account on current clinical trials using SVF/ASCs

Conditions	Status	Phase	Treatment/mode of infusion	NCT number
Graft-versus-host disease; chronic and expanded Graft-versus-host disease; immune system diseases	Recruiting	Phase 1 Phase 2	Conventional treatment and intravenous infusion of allogenic MSC	NCT01222039
Autoimmune diseases; immune system diseases; demyelinating diseases; nervous system diseases; demyelinating autoimmune diseases, CNS	Recruiting	Phase 1 Phase 2	Intravenous infusion of autologous MSCs	NCT01300598
Degenerative arthritis	Recruiting	Phase 1 Phase 2	Intra-articular infusion of autologous MSCs	NCT01300598
Buerger's disease	Recruiting	Phase 1 Phase 2	Intramuscular infusion of autologous MSCs	NCT01302015
Articular cartilage lesion of the femoral condyle	Not yet recruiting	Phase 1 Phase 2	Implantation of autologous ASC or chondrocytes	NCT01399749
Osteoporotic fractures	Not yet recruiting	Phase 2	Cellularized and acellular composite graft augmentation	NCT01532076
Soft tissue mass removal	Completed	–	Liposuction	NCT01399307
Progressive hemifacial atrophy; Romberg's disease	Completed	Phase 2	Autologous MSC transplantation	NCT01309061
Leukemia; Hodgkin's lymphoma; non-Hodgkin's lymphoma; myelodysplastic syndrome	Active, not recruiting	–	Questionnaires, laboratory tests, abdominal MRI	NCT00510315
Rectovaginal fistula	Recruiting	Phase 1 Phase 2	Intralesional injection of cell suspension	NCT01548092
Anal fistula	Unknown	Phase 2	Nonsurgical autologous implant of ASCs	NCT00115466
	Completed	Phase 3	Intralesional injection of ASCs with fibrin glue	NCT00475410
Complex perianal fistula	Recruiting	Phase 2	Autologous cultured adipose-derived stem cells	NCT01314092
	Completed	–	Intralesional injection of ASCs with fibrin glue	NCT01020825
Crohn's fistula	Recruiting	Phase 1	Infusion of allogenic ASCs	NCT01440699
Crohn's disease	Recruiting	Phase 1 Phase 2	Autologous MSCs	NCT01300598
Frailty syndrome	Recruiting	Phase 1 Phase 2	Intravenous injection of MSCs	NCT01501461
Diabetes mellitus type II	Recruiting	Phase 1 Phase 2	Intrapancreatic injection; intravenous injection of the ASCs	NCT01453751
	Unknown	Phase 1 Phase 2	Intravenous injection of autologous activated SVF	NCT00703612
Diabetes mellitus type I	Unknown	Phase 1 Phase 2	Intravenous injection of autologous activated SVF	NCT00703599
Diabetes; limb ischemia	Recruiting	Phase 1 Phase 2	Intra-arterial infusion of autologous MSCs	NCT01079403
Lower limb ischemia	Recruiting	Phase 1 Phase 2	ASC-coated ePTFE vascular graft	NCT01305863
Parkinson's disease	Recruiting	Phase 1 Phase 2	Harvesting and implantation of ASCs (catheter injection)	NCT01453803
Brain lesion (general)	Recruiting	Phase 1 Phase 2	ASCs delivered via catheter into the internal carotid artery and intravenously	NCT01453777

(continued)

Table 10.3 (continued)

Conditions	Status	Phase	Treatment/mode of infusion	NCT number
Multiple sclerosis	Recruiting	Phase 1 Phase 2	ASCs delivered via intravenous injection and intrathecally	NCT01453764
Non-ischemic congestive heart failure	Recruiting	Phase 1 Phase 2	ASCs delivered intramyocardially and intravenously	NCT01502501
Stroke	Recruiting	Phase 1 Phase 2	ASCs delivered into the internal carotid artery and intravenously	NCT01453829
Renal failure	Recruiting	Phase 1 Phase 2	ASCs delivered via catheter into the renal artery and intravenously	NCT01453816
Depressed scar	Completed	Phase 2 Phase 3	Autologous cultured adipocytes via subcutaneous injection	NCT00992147
Lipodystrophy	Unknown	Phase 1	Lipoinjection enriched with ASCs	NCT00715546

immunoregulatory properties similar to those of autologous or allogenic MSCs [183, 184]. This opens new perspectives to the use of adipose MSCs for treating GVHD.

Autoimmune Diseases

Immunomodulatory properties appear to be an intrinsic property of ASC and thus present an attractive basis for the therapy of autoimmune and inflammatory diseases by systemic infusion. Treatments for autoimmune diseases were initiated in patients after other treatment options were exhausted. Recently, ASCs have been explored for its potential immunoregulation in autoimmune disease systemic lupus erythematosus (SLE) [185]. It is suggested that immunomodulation of ASCs was achieved by partially suppressing the number and capability of Th17 lymphocytes, indicating that ASCs could be employed as therapeutic tools for the autoimmune diseases. There is still a limited understanding of the modes of action of stem cells during the treatment of autoimmune diseases. Thus, systemic infusion of autologous stem cells might offer promise for better management of a wide spectrum of autoimmune diseases, independent on patient's age if further research on modes of action is explored.

Subcutaneous Adipose Stem Cell in Clinical Trial

Human subcutaneous adipose stem cells are abundant, accessible, and reliable source of therapeutic applicability in preclinical/clinical studies in diverse fields. It is rapidly increasing and gaining importance in recent years due to their ability to readily be expanded and their capacity to undergo multilineage differentiation and positive experimental data accumulated from the studies outlined above. The safety and efficacy of ASCs for tissue regeneration or reconstruction are currently under assessment in clinical trials. Although the clinical trials of BMSC crossed 500 in numbers completed or underway, clinical trials using ASC are less in number. However, the number of trials has risen rapidly from a total of 9 in December 2009, 18 by May 2010, to 32 in April 2012, investigating the efficacy in treating conditions such as type I and II diabetes, fistulas, cardiovascular disease, limb ischemia, depressed scar, lipodystrophy, and so on (http://clinicaltrials.gov) (Table 10.3). Furthermore, there are a limited number of currently ongoing phase III clinical trial investigating autologous ASCs in repairing perianal fistulas and depressed scar. Although the full publication of data from many of these trials is pending, early information from selected trials has been presented recently.

Conclusion

Regenerative medicine involves a multidisciplinary effort to replace or repair diseased tissue. Stem cell therapy and tissue engineering are the key components of regenerative medicine. However, both are linked by one common aim to deliver safe, effective, and consistent therapy to patients. Subcutaneous adipose depots are accessible, abundant, and replenishable and had accomplished these objectives, thereby providing a potential source as adult stem cell for treating a wide range of therapeutics. Several groups have demonstrated that mesenchymal stem cells within the human subcutaneous adipose tissue possess self-renewing ability and display multilineage developmental plasticity in vitro and in vivo. It is now becoming increasingly accepted that the likely predominant mechanism of action of ASC is survival, proliferation, and differentiation into tissue-specific stem cells, which then get incorporated into diseased tissues and repopulate the area with a healthy cell population thereby exerting an appreciable effect and promoting endogenous repair process.

Although our understanding of ASC biology is gradually increasing in recent years, many challenges lie ahead in aspects of the wealth of knowledge of all normal process and steps involving cell differentiation and tissue formation, before we can realize the full potential of these cells for clinical and therapeutic applications.

This chapter has highlighted some of the aspects of current important research investigations and key areas for future investigation which include the catalogue of cell surface marker characterization that enables the isolation of pure population of ASCs, prerequisites for ex vivo expansion studies, and optimization of culture condition, multilineage differentiation potential, and current registered clinical trials using ASC. The advances that have been observed with ASC have provided evidence of their great potential and applicability in cell therapy, as well as in the enhancement of healing process. Moreover, their reproducibility, easily handled characteristics, accessibility, and facility of being obtained in a relatively large quantity make them an ideal source of transplantation and a boost for regenerative medicine.

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11 Expanded Adipose Tissue-Derived Stem Cells for Articular Cartilage Injury Treatment: A Safety and Efficacy Evaluation

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Introduction

Cartilage injuries are a common clinical problem in humans, especially for those over 40 years of age, and often lead to osteoarthritis in patients if they are not treated appropriately. Osteoarthritis is a chronic, degenerative process characterized by progressive cartilage deterioration, subchondral bone remodeling, a loss of joint space, marginal osteophytosis, and loss of joint function [78]. Presently, cartilage injuries are mainly treated with the aim of alleviating symptoms, reducing pain, and controlling inflammation, such as with drugs [5, 16, 20, 62] or injections of hyaluronic acid [10, 37, 72]. However, these therapies have limited efficacy and offer no prevention against progressive degeneration of the joint [69].

Two to three years ago, stem cell therapy was considered a promising strategy for the treatment of injured articular cartilage and osteoarthritis. Many researchers have used stem cells from various sources to treat osteoarthritis, with differing success rates. In all cases, however, mesenchymal stem cells (MSCs) were deemed the most suitable source of stem cells for treatment. MSCs are multipotent cells that can differentiate into bone, cartilage, and fat, among others [63]. MSCs have been isolated from numerous sources, such as the bone marrow [59], adipose tissue [19], umbilical cord blood [65], banked umbilical cord blood [60], umbilical cord [23], Wharton's jelly of the umbilical cord [58], placenta [61], and dental pulp [73]. However, MSCs from bone marrow [49, 14, 38] and adipose tissue [7, 26, 56] are the two main sources of stem cells for degenerative cartilage treatment, as they offer an easy mode of cell collection. Furthermore, adipose tissue has been touted as the best source of MSCs for disease treatment.

MSCs from adipose tissue, or ADSCs, have been extensively investigated in preclinical studies for the treatment of cartilage injuries and osteoarthritis in animal models such as dog [1, 2, 30], rabbit [75], horse [26], rat [45], mouse [74], and goat [53]. The results from these studies showed evidence of neo-cartilage proliferation from ADSC transplantation and have further encouraged human clinical trials for the treatment of osteoarthritis. For instance, Park [56] showed significant positive changes for all patients transplanted with ADSCs. Various Phase I and II clinical trials using ADSCs for osteoarthritis or degenerative cartilage have been undertaken (NCT01300598, NCT01585857, NCT01399749); however, almost all of these studies have used non-expanded ADSCs derived from the stromal vascular fraction (SVF) of adipose tissue.

The SVF, however, has several limitations; in particular, there are very few ADSCs in the SVF. Recent studies have attempted to culture and enrich ADSCs from SVF prior to their transplantation, have eliminated the xenogenic components in culture medium, or have developed serum-free media conditions for ADSC culture [17, 79]. Although these studies have demonstrated the benefits of ADSCs for osteoarthritis, especially in the knee joint, up until now, a comprehensive study that evaluates the safety and efficacy of ADSCs in the treatment of degenerative cartilage disease has not been performed. As such, this research aims to evaluate the safety and efficacy of expanded ADSC transplantation for articular cartilage injury in a mouse model and investigate the role of ADSCs in cartilage joint regeneration.

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Isolation and Characterization of ADSCs

Stromal Vascular Fraction Cell Isolation from Adipose Tissue

Stromal cells were first isolated from the abdominal adipose tissue of ten, consenting healthy donors. From each patient, approximately 40–80 mL of lipoaspirate was collected in two 50-mL sterile syringes. All procedures and manipulations were approved by our Institutional Ethical Committee (Laboratory of Stem cell Research and Application, University of Science, VNU-HCM, VN) and the Hospital Ethical Committee (Ho Chi Minh City Medicine and Pharmacy University Hospital, HCM, VN). The syringes were held in a sterile box at 2–8 °C and immediately transferred to the laboratory. The SVF was isolated using an ADSC Extraction kit, according to manufacturer's instructions (GeneWorld, HCM, VN). Briefly, 80 mL of lipoaspirate was flushed into a sterile disposable 250-mL conical centrifuge tube (2602A43, Corning). The adipose tissue was washed twice using phosphate buffer saline (PBS) by centrifugation at 400 × g for 5 min at room temperature. Next, the adipose tissue was digested using SuperExtract Solution (1.5 mg of collagenase per mg of fat tissue) at 37 °C for 30 min, with agitation at 5-min intervals. The suspension was centrifuged at 800 × g for 10 min, and the SVF was obtained as a pellet. The pellet was washed twice with PBS to remove any residual enzyme and resuspended in PBS to determine cell quantity and viability using an automatic cell counter (NucleoCounter, ChemoMetec). The results showed isolation of $0.95 \pm 0.13 \times 10^6$ stromal cells per 1 g of adipose tissue, with a viability of 76.1 ± 4.86 % cells ($n = 10$).

ADSC Expansion

The SVF cells were cultured to enrich for and expand the number of ADSCs. SVF cells were suspended in serum-free DMEM/F12 medium, supplemented with 1X antibiotic-mycotic (Sigma-Aldrich, St Louis, MO) and 2 % human PRP (platelet-rich plasma), which was derived from the same donor as the adipose tissue as described previously [42]. Cells were cultured at 37 °C with 5 % CO_2, and the media changed twice per week. The samples were cultured with 100 % success rate (10/10). Spindle-shaped or fibroblast-like cells appeared in culture after 24 h (Fig. 11.1a). The cells were incubated for a further 24 h to increase the proportion of adherent cells prior to the first medium change. From day 3, cells rapidly proliferated and co-cloned on day 7 (Fig. 11.1b, c). When cells had reached 70–80 % confluence, they were subcultured using 0.25 % trypsin/EDTA (GeneWorld, HCM, VN). To determine cell quantity at the first passage, cell counts were obtained and analyzed in correlation with the original adipose tissue weight. The results revealed 107.37×10^3 adherent cells from 10^6 nucleated cells in 1 g of adipose tissue. Therefore, the adherent cell population accounted for 11.26 % of the nucleated cells. From these results, we determined the population doubling time using the algorithm provided by http://www.doubling-time.com [67] to calculate the culturing time required for producing the large number of cells required in the clinic. The population doubling time of ADSCs was determined as 48 ± 4 h.

Expanded ADSCs Maintained the MSC Phenotype

ADSCs at the tenth passage were first examined for their immunophenotype using flow cytometry, according to previously published protocols [60]. Briefly, cells were washed twice in Dulbecco's modified PBS (D-PBS) supplemented with 1 % bovine serum albumin (Sigma-Aldrich, St Louis, MO). Cells were stained for 30 min at 4 °C with anti-CD14-FITC, anti-CD34-FITC, anti-CD44-PE, anti-CD45-FITC, anti-CD90-PE, and anti-CD105-FITC monoclonal antibodies (BD Biosciences, Franklin Lakes, New Jersey). Stained cells were analyzed using a FACSCalibur flow cytometer

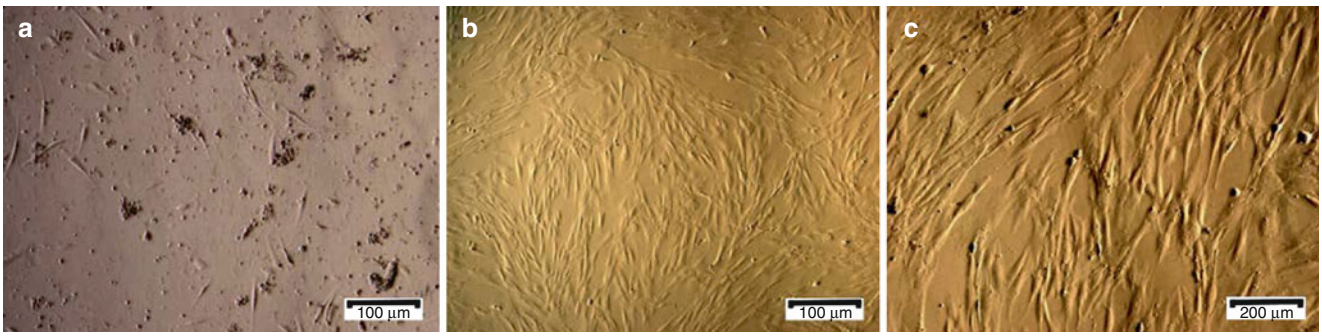

Fig. 11.1 ADSCs were cultured in serum-free medium. The stromal vascular fraction (SVF) from the abdominal adipose tissue of healthy donors was isolated and cultured in DMEM containing platelet-rich plasma in 37 °C, 5 % CO_2 (representative images for cultures at 24 h (**a**), 7 days (**b**), the tenth subculture (**c**))

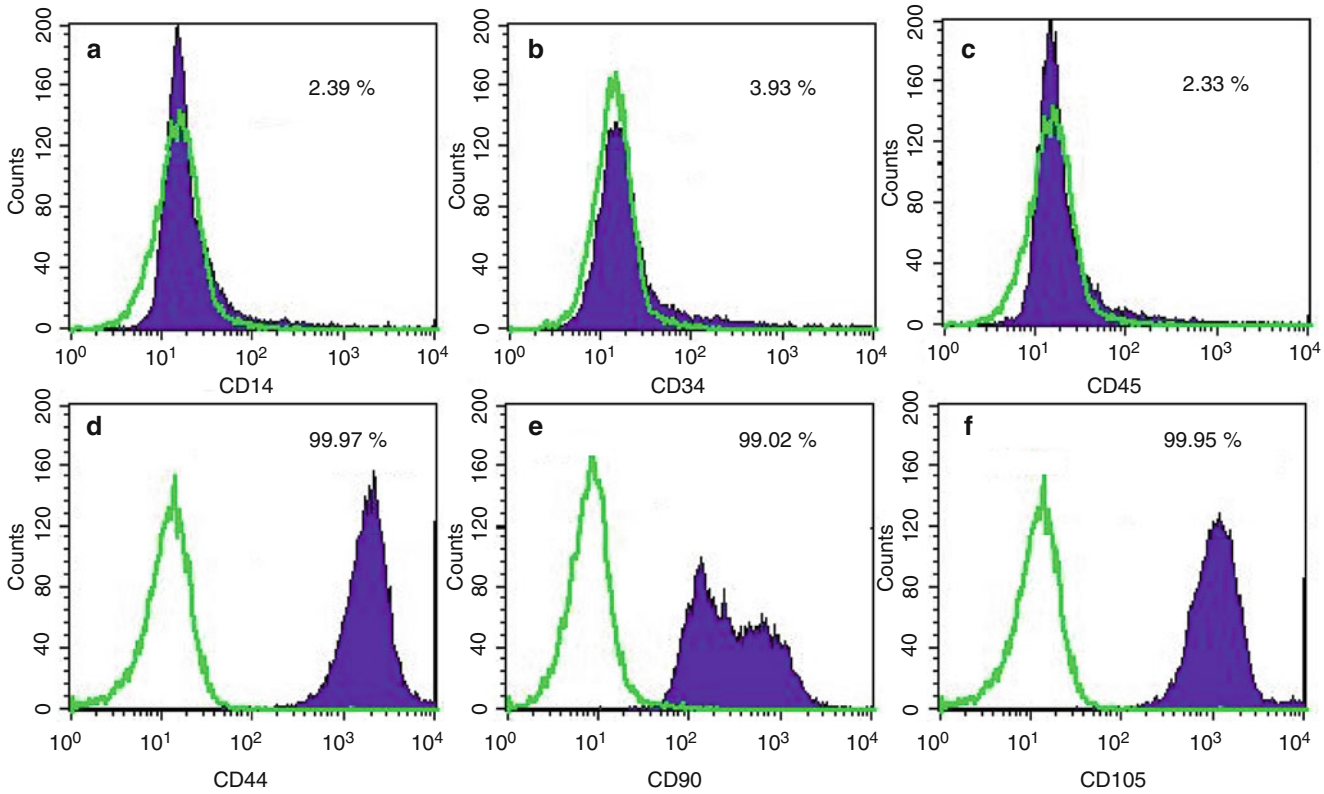

Fig. 11.2 Mesenchymal stem cell (MSC)-specific marker expression of ADSCs. ADSCs were positive for MSC markers—CD44 (**d**), CD90 (**e**), and CD105 (**f**)—and negative for markers specific to other lineages: CD14 (**a**), CD34 (**b**), and CD45 (**c**)

(BD Biosciences). Isotype controls were used for all analyses. The results showed that ADSCs at the tenth passage maintained a MSC-specific immunophenotype, with more than 99 % positive staining for CD44, CD90, and CD105 (Fig. 11.2d–f) and negative staining for CD14, CD34, and CD45 (Fig. 11.2a–c) and with less than 4 % of the cells positive for these hematopoietic markers.

Expanded ADSCs Could Be Induced to Differentiate into Adipocytes and Chondrocytes

We next tested the differentiative capacity of expanded ADSCs into adipocytes or chondrocytes. For adipocytic differentiation, ADSCs at passage 10 were subcultured into 12-well tissue culture plates (Falcon, France), and differentiation was induced as per previously published protocols [17]. Briefly, ADSCs were incubated for 3 days in adipogenic-inducing medium (ADSC expansion medium, supplemented with 1-μM dexamethasone, 450-μM IBMX, and 60-μM indomethacin (Sigma-Aldrich, St Louis, MO). IBMX was then removed from the medium and the cells were cultured for a further 19 days. Adipogenic differentiation was evaluated using oil red O staining to determine the presence of lipid droplets (stained red) in the cytoplasm [43]. After adipogenic induction, we observed the accumulation of lipid droplets in the cytoplasm of ADSCs (Fig. 11.3b) as compared with cells before induction (Fig. 11.3a) that were positive with oil red O staining (Fig. 11.3c). These results demonstrated that ADSCs at the tenth passage are capable of differentiating into adipocytes.

Chondrocytic differentiation was also induced using previous published procedures [18, 35, 80]. Briefly, ADSCs at passage 10 were cultured in chondrogenic culture medium (ADSC expansion medium supplemented with 1X insulin-transferrin-selenium supplement (ITS), 0.15-mM ascorbate 2-phosphate, 100-nM dexamethasone, and 10-ng/ml recombinant human (rh)-transforming growth factor (TGF)-β1 (Sigma-Aldrich, St Louis, MO). Chondrocytic differentiation was evaluated by RT-PCR for markers specific to chondrocytes, with all experiments performed in triplicate, and a *P*-value of ≤ 0.05 was considered statistically significant. Analysis was performed using Statgraphics software (v 7.0; Statgraphics Graphics System, Warrenton, VA). The results showed that after 21 days in inducing media, ADSCs differentiated into chondrocytes, with positive expressions of collagen type I (Col IA2), collagen type II (Col II), and cartilage oligomeric protein (COMP) at levels that were 20.10 ± 8.32, 1.55 ± 0.92, and 5.09 ± 3.08 times higher as compared with undifferentiated ADSCs, respectively (Fig. 11.6; *note*: refer to in vitro (blue bar graph) results for cells).

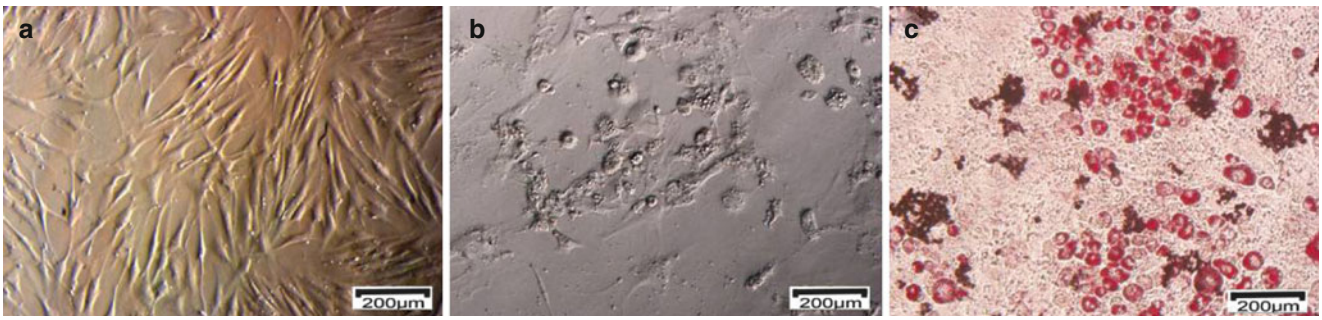

Fig. 11.3 Differentiation potential of ADSCs into adipocytes. Expanded ADSCs (**a**) became rounded following adipocyte induction and accumulated lipid droplets in the cytosol (**b**). These cells were positive (*red*) for oil red O staining (**c**)

Safety and Efficacy of ADSCs in Cartilage Regeneration

Expanded ADSCs Show No Indication of Tumorigenic Potential

To demonstrate that expanded ADSCs are safe for transplantation, we measured the tumorigenesis-related gene expression and examined the in vivo tumor formation potential of ADSCs. For gene expression evaluation, total RNA was extracted from tenth passage ADSCs using TRIzol, according to the manufacturer's guidelines (Sigma-Aldrich, St Louis, MO). Briefly, 1-ml TRI Reagent was added into each 1.5-ml tube and mixed by trituration. Tubes were then centrifuged at 3,000 rpm, at 22 °C for 5 min. The supernatant was collected and transferred into a new tube and mixed with 200 μl of chloroform. The tubes were incubated at 4 °C for 5 min before centrifugation at 12,000 rpm, 4 °C for 15 min. RNA was precipitated by isopropyl alcohol (500 μl) at room temperature for 10 min. ADSCs were analyzed for the expression of certain genes related to cancer cell markers or embryonic stem cells—*Oct-3/4* and *Nanog*—using SYBR Real-Time RT-PCR one tube one step (Sigma-Aldrich, St Louis, MO). The forward and reverse primers for gene amplification are listed in Table 11.1. The results showed that non-expanded ADSCs as well as expanded ADSCs at the tenth passage did not express the *Oct-3/4* or *Nanog* (data not shown).

Next, the tumorigenic potential of the cells was assessed in a tumor formation assay using 5- to 6-week-old non-obese diabetic NOD/SCID mice (*NOD.CB17-Prkdcscid/J*, Charles River Laboratories). All mice were housed in clean cages and maintained according to institutional guidelines on animal welfare approved by the Local Ethics Committee of the Stem Cell Laboratory, University of Science (VNU-HCM, VN). Mice were injected with 10^7, 10^6, or 10^5 cells under the skin, with three mice per group. PBS injections were used in the control mice. The mice were followed up for 3 months for the detection of tumors. The results showed that all of the mice (9/9) were tumor-free after 3 months as well as the control group of mice (data not shown).

Expanded ADSCs Can Recover the Articular Cartilage Injury in Mice Model

To evaluate the effects of expanded ADSCs on osteoarthritis, we used a mouse model of articular cartilage injury. All procedures were approved by the Local Ethics Committee of the Stem Cell Research and Application Laboratory, University of Science (VNU-HCM, VN). The articular cartilage injury was induced by joint destruction to the hind limbs of NOD/SCID mice using a fine 32-gauge needle. Briefly, nine mice were anesthetized using ketamine (40 mg/kg) and then subjected to hind limb joint destruction, with one additional mouse used as a positive control (uninjured). Of these nine mice, five were randomly allocated to the treatment group and four to the negative control group. Six hours after injury, 9/9 mice were recorded as being unable to use their hind limbs or control their movement on the table. The mice were then anesthetized and injected with either expanded ADSCs (treatment) or PBS (negative control). In the treatment group, 2×10^6 expanded ADSCs suspended in 200-μl PRP were injected into the knee joint via two doses, with a 10-min interval between injections. Mice in the negative control group received PBS injections.

For each mouse, the hind limb movement on the table was evaluated daily. After 45 days, all mice were euthanized, and their hind limbs used for histological analysis and further experiments. The samples were fixed in 10 % formalin, decalcified, sectioned longitudinally, and stained with hematoxylin and eosin (HE) (Sigma-Aldrich, St Louis, MO). Using HE-stained slides, three parameters for the knee joints were examined: the area of damaged cartilage (%), the area of neo-cartilage (%), and the number of neo-cartilage cell layers. The damaged cartilage area was determined by mature cartilage that was lost in compare to control. Data were analyzed using Statgraphics software (v 7.0;

Table 11.1 Forward and reverse primer sequences

Gene	Accession no.	Sequence
Oct-3/4	NM_002701.3	F:5′-GGAGGAAGCTGACAACAATGAAA-3′
		R:5′-GGCCTGCACGAGGGTTT-3
Nanog	NM_028016.2	F:5′-ACAACTGGCCGAAGAATAGCA-3′
		R:5′-GGTTCCCAGTCGGGTTCAC-3
Collagen I	NM_000088	F: 5′-TGACGAGACCAAGAACTG-3′
		R:5′-CCATCCAAACCACTGAAACC-3′
Collagen II	NM_001844	F: 5′-TTCCCCAGGTCAAGATGGTC-3′
		R: 5′-CTTCAGCACCTGTCTCACCA-3′
COMP	NM_000095	F: 5′-CAGGACGACTTTGATGCAGA-3′
		R: 5′-AAGCTGGAGCTGTCCTGGTA-3′
GAPDH	NM_002046	F: 5′-GGGCTGCTTTTAACTCTGGT-3′
		R: 5′-TGGCAGGTTTTTCTAGACGG-3

Abbreviations: *Col IA2* collagen type I, *Col II* collagen type II, *COMP* cartilage oligomeric matrix protein

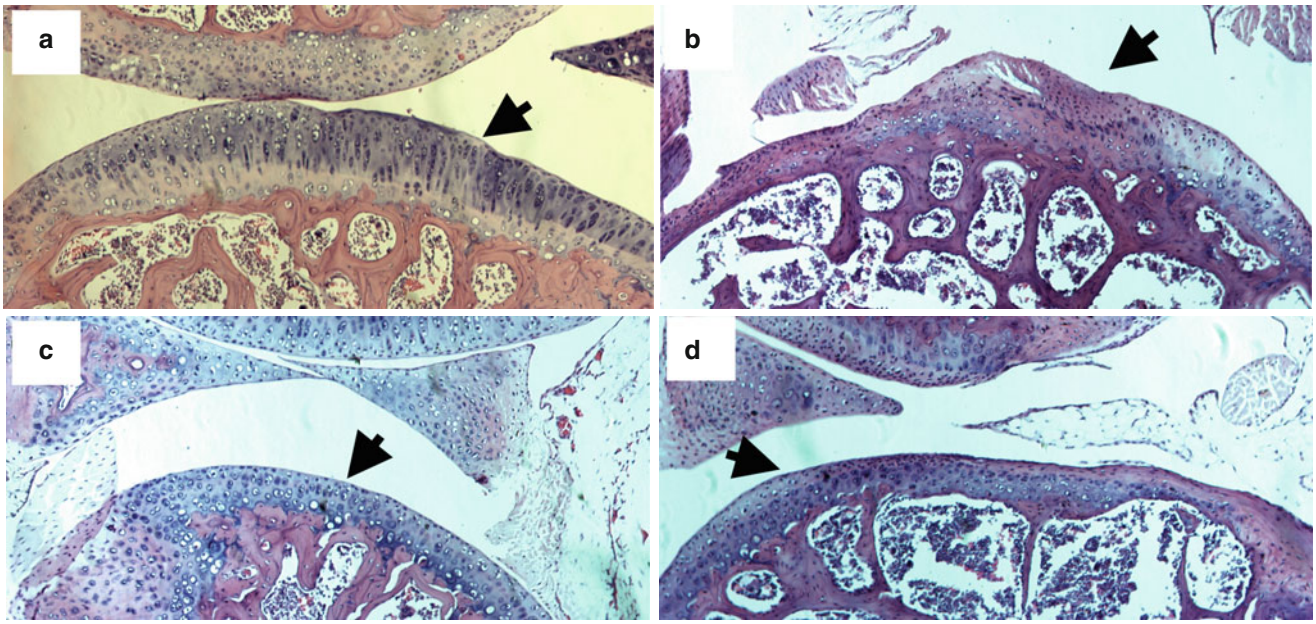

Fig. 11.4 Recovery of mouse knee joints on HE stained sections. Results from HE staining of articular cartilage slides showed that articular cartilage of treated mouse (**b**) rapidly recovered, as compared with the negative control (**c**) that only injected by PRP; and (**d**) non-injected. There was evidence of neo-cartilage proliferation at the articular cartilage margin in the treated mice, and the thickness of the cartilage layer of the treated mice was similar to that of a normal, uninjured (positive control) mouse (**a**)

Statgraphics Graphics System, Warrenton, VA). The results showed a significant difference among the treated and negative control groups, especially in regard to the time until which the mice could control their hind limb movement as well as the regeneration of the joint cartilage. The time until recovery of hind limb movement reduced from 36.5 ± 2.08 days in the negative control (PBS) mice to 22.4 ± 2.97 days in the treated (expanded ADSC) mice. For mice in the treatment group, the histological analysis showed that the mean area of damaged joint cartilage was 70 %, with 40 % neo-cartilage formed after 45 days ($n = 5$). This neo-cartilage layer had 12.8 layers of neo-chondrocytes. By comparison, in the negative control group, the mean area of damaged joint cartilage was 53.13 %, but there was only 12.5 % neo-cartilage formed after 45 days, with 12 layers of neo-chondrocytes (Fig. 11.4).

Expanded ADSCs Existed and Differentiated into Chondrocytes at Murine Joint Sites

To determine the persistence of grafted cells at articular cartilage healing sites, ADSCs were transfected with a lentivirus carrying *gfp* (Santa Cruz Biotechnology) and selected by culture in expansion medium supplemented with 10-μg/mL puromycin dihydrochloride (Sigma-Aldrich, St Louis, MO)

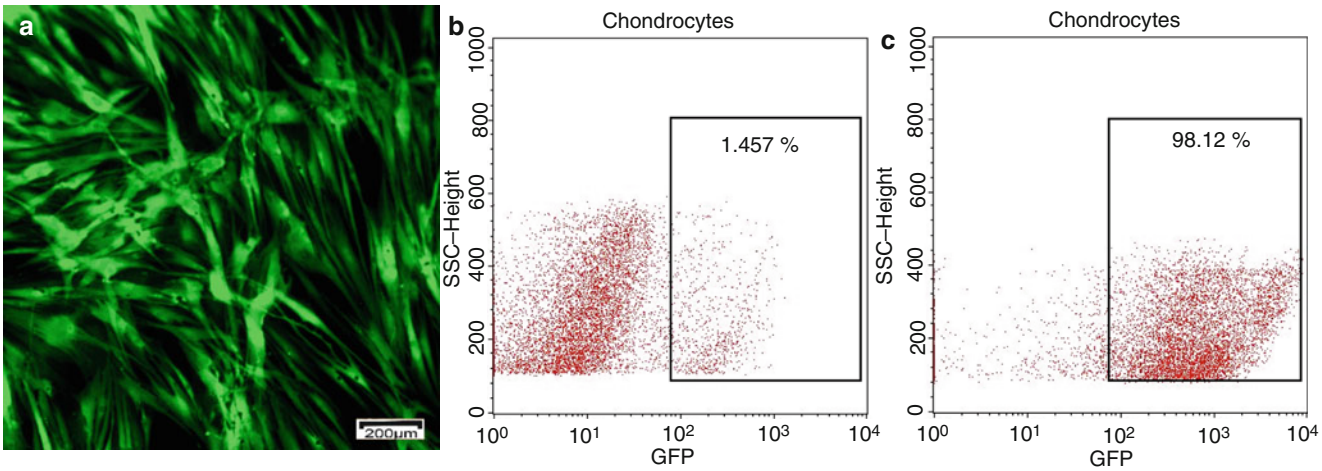

Fig. 11.5 Existence and differentiation of grafted ADSCs. (**a**) GFP stable expression of ADSCs after *gfp* transgenesis. (**b**) Existence of ADSCs in murine joint was recognized by flow cytometry. (**c**) The grafted ADSCs exhibited high purity after sorting that was used for further experiments

for 1 week, as per the manufacturer's recommendations. GFP-stably expressing ADSCs were used to treat mice with articular cartilage injury, as described in section "Expanded-ADSCs can recover the articular cartilage injury in mice model" (Fig. 11.5a). There were three NOD/SCID mice used in this experiment. After 45 days, we collected the articular cartilage, isolated the chondrocytes following the procedure of Gosset et al. [29], and confirmed the existence of grafted cells by flow cytometry using flow cytometer FACSCalibur (BD Bioscience, USA). The results showed that after 45 days, grafted ADSCs persisted in the articular cartilage. GFP-positive cells accounted for 0.746 ± 0.432 % (ranging from 0.123 % to 1.457 %) ($n = 3$) of the total cell number (Fig. 11.5b).

Next, we evaluated the in vivo differentiation status of these grafted ADSCs. GFP-positive grafted cells were sorted based on the GFP signal using a cell sorter FACSJazz system (BD Bioscience, USA). The purity of these sorted cell populations was confirmed by flow cytometer FACSCalibur. The result showed a high purity of the sorted cells (98.12 %) (Fig. 11.5c). The RNA from these sorted cells was then isolated by TRI Reagent, as described in section "Expanded-ADSCs show no indication of tumorigenic potential," and the expression of chondrocyte differentiation-specific genes was determined. The expression of genes of interest was normalized to GAPDH and analyzed by $2^{-}\Delta\Delta^{C}T$ method [48]. All experiments were performed in triplicate. A *P*-value of ≤ 0.05 was considered to be significant. Data were analyzed using Statgraphics software (v 7.0; Statgraphics Graphics System, Warrenton, VA). The results showed that grafted ADSCs expressed *Col IA2*, *Col II*, and *COMP* at 84.10 ± 38.56, 2.04 ± 0.24, and 26.72 ± 6.15 times higher than the levels in non-grafted ADSCs, respectively, as well as in vitro differentiated ADSCs (Fig. 11.6). High expression of these genes demonstrated that grafted ADSCs changed their gene profile toward a chondrocytic phenotype or differentiated in vivo into chondrocyte phenotype.

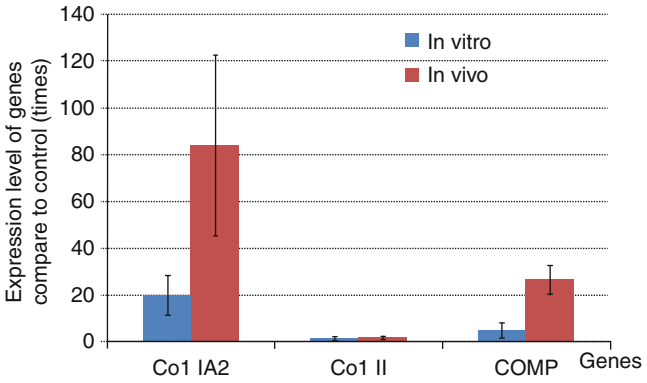

Fig. 11.6 Gene expression of Col IA2, Col II, and COMP in differentiated ADSCs in vitro and in vivo. *Col IA2* and *COMP* expressions in vivo were higher than in vitro conditions ($P < 0.05$)

Discussion

Stem cell therapy is considered a promising therapy for degenerative diseases. MSCs are the most commonly used stem cell type for solid tissue regeneration. MSCs were first isolated from bone marrow and originally named bone marrow stromal cells [25]. MSCs hold enormous differentiative potential, especially for the differentiation of cells into those from the mesenchymal lineages, including bone, cartilage, and fat. Autologous MSC transplantation is considered a safe and efficient source of MSCs for some degenerative diseases. Adipose tissue has recently been recognized as a

convenient, alternate source of MSCs. ADSCs are found in abundance in adipose tissue [27] and can be cultured for an extended duration in vitro, without undergoing senescence [32]. Similar to MSCs from other sources, ADSCs also successfully differentiate into adipocytes, osteoblasts, and chondrocytes and can transdifferentiate into neurons and myocytes [32, 34, 39, 52, 68, 77, 83]. As such, ADSCs have been described as a feasible source of stem cells for the repair of injured articular cartilage. However, few studies have investigated the roles of grafted, expanded ADSCs at the injury site. Here, we performed a comprehensive study to investigate the safety and efficacy of expanded ADSCs using a murine articular cartilage injury model.

In the first experiment, we isolated the stromal vascular fraction (SVF) from adipose tissue. Comparable with other studies, we isolated $0.95 \pm 0.13 \times 10^6$ stromal vascular cells per 1 g of adipose tissue, with 76.1 ± 4.86 % viable cells ($n = 10$). From these stromal vascular cells, we collected $107{,}368 \times 10^3$ adherent cells. This result was higher than some previous reports, where they isolated approximately 5×10^3 stem cells from 1 g of adipose tissue [24, 40]. This result may suggest that adipose tissue offers a richer source of MSCs than that from an equivalent amount of bone marrow.

Until now, numerous studies have investigated the efficacy of ADSC transplantation in animal models, as well as in humans. Most studies have shown that ADSC transplantation significantly improved neo-cartilage proliferation. However, the efficacy was mainly dependent on the quantity of the injected ADSCs. Consequently, in vitro ADSC proliferation is vital for successful results. Serum often contains harmful xeno-agents, especially animal viruses. Therefore, in this research, we enriched the proportion of ADSCs from the SVF using fetal bovine serum (FBS)-free medium and instead supplemented the media with PRP derived from the same donor as the adipose tissue. PRP contains several different growth factors and cytokines that stimulate the cells to proliferate [4, 81]. So PRP was used as alternative source of serum to culture MSCs from the bone marrow [28], dermal fibroblast [36], and ADSCs [36, 41]. The results showed a high rate of ADSC proliferation in this medium. After the tenth passage, ADSCs expressed MSC-specific markers (CD44, CD90, and CD105) and were negative for hematopoietic cell markers (CD14, CD34, and CD45). Furthermore, these expanded ADSCs could be differentiated into adipocytes and chondrocytes in vitro and overall satisfied the minimal criteria for defining human MSC [15], similar to the phenotypes of ADSCs cultured in FBS medium [39, 54, 57, 64, 82, 84] or defined serum-free medium [3, 17, 70, 79]. Moreover, the ADSC doubling time was quicker than previously reported for ADSC cultured in FBS medium: indeed, the doubling time of ADSCs was 48 ± 4 h as compared with 70 h [9] or 2–4 days [34, 50].

While in vitro MSC expansion provides sufficient MSC cell counts for transplantation, there is an element of risk involved, especially with the possibility of introducing tumor-inducing mutations. Therefore, we limited ADSC expansion to just ten passages. This number was chosen based on the time required to obtain sufficient cell counts while maintaining the stemness of the cells, without inducing tumorigenesis. This was confirmed by a lack of *Oct-3/4* and *Nanog* gene expressions. *Oct-3/4* and *Nanog* expressions are involved in the self-renewal of undifferentiated embryonic stem cells [8, 51, 55], and their proteins have been implicated in the tumorigenesis of adult germ cells [33], with ectopic expression causing tumor formation such as oral squamous cell carcinoma [12, 76], lung cancer [11], breast cancer [47], and glioma [31]. We further confirmed this inability to form tumors in vivo by injecting the cells under the skin of NOD/SCID mice. From these experiments, we conclude that expanded ADSCs are safe for transplantation.

Once we confirmed the safety of ADSCs, we next examined their efficacy in healing injured articular cartilage. The results showed that ADSCs differentiated into chondrocytes and expressed some of the functional genes related to chondrogenesis, including *Col I*, *Col II*, and *COMP*. The expression of these genes was also confirmed in vitro under directed chondrocytic induction. Furthermore, ADSC transplantation reduced the healing time to nearly one-half that seen in normal repair (22.4 ± 2.97 days vs 36.5 ± 2.08 days in treated vs control groups, respectively). In this model, joint cartilage was destroyed using a fine gauge needle, and the mice were assigned into two groups. In the treated group, the mice showed 70 % destruction of the cartilage joint surface, whereas only 53 % destruction was observed in the control mice. This difference was dissimilar effects of fine gauge needle among mice. After 45 days, we observed up to 40 % neo-cartilage in the treated group, but only 12.5 % neo-cartilage in the control group. Interestingly, this demonstrates that mice have an excellent capacity to regenerate part of the injured cartilage without the contribution of foreign agents but that this can be accelerated by ADSC transplantation and their subsequent differentiation. More importantly, the articular cartilage in both the treated and control mice matured to nearly the same extent, with 12.8 layers vs 12 layers in treated and control groups, respectively. This indicates that ADSCs naturally integrated into the articular cartilage during self-renewal in the joint microenvironment. No scarring or tumor formation was observed at the grafted sites.

Our findings support those of many previous published reports in mice, rats, pig, dog, etc. In a collagenase-induced articular injury mice model, Ter Huurne et al. [74] showed that the degree of damage was reduced by nearly 50 % with ADSC transplantation, as compared with that in control mice after 42 days. They showed that ADSC transplanta-

tion protected and repaired the joint cartilage to such an extent that only 25 % of ADSC-treated mice presented with knee joint dislocation, as compared with 88 % in the control group [74]. In agreement, Dragoo et al. [85] showed that autologous ADSCs can reconstruct articular surface defects in rabbits, with 100 % (12/12) of rabbits showing a hyaline-like cartilage, as compared with only 8 % (1/12) in the control group ($p < 0.001$) (Dragoo et al. 2007). The efficacy of ADSC transplantation, however, may arise from other roles of the cells. Similar to MSCs derived from the bone marrow, ADSCs have an anti-inflammatory capacity [13, 6, 71, 46] and are able to inhibit graft versus host disease (GVHD) [44, 66]. Indeed, ADSC transplantation can successfully treat steroid refractory GVHD [21, 22]. As such, the accelerated cartilage repair presented in this study may also be due to the non-chondrogenic functions of ADSCs.

Conclusion

Adipose tissue provides a rich source of MSCs, with a high proliferative capacity that maintains its stemness for at least up to ten rounds of subculture. ADSCs expanded in serum-free medium supplemented with PRP offer a safe source of stem cells, suitable for clinical use. However, although expanded ADSCs appear to be safe and effective for articular cartilage repair, this research only evaluated tumorigenesis of grafted cells and tissues as well as single, intra-articular injections. There are possible side effects, such as fever and myalgia, in the use of expanded ADSCs and multiple transplantations, and we are still unsure of the safety of repeated intra-articular injections. In combination with results from previous preclinical and clinical trials, we propose that ADSC transplantation will provide benefit effects for cartilage regeneration, with relative safety. After long-term proliferation, ADSCs neither express genes related to tumorigenesis nor induce tumor formation in NOD/SCID mice. Indeed, expanded ADSC transplantation can regenerate the neo-cartilage in an articular cartilage injury model in mice. Grafted cells integrated with the host cartilage and differentiated into neo-chondrocytes. The self-renewal and differentiative potential of grafted ADSCs led to the regeneration of injured cartilage and reduced the healing time to half that which is observed under normal conditions. This research demonstrates comprehensively the safety and efficacy of ADSC therapy for degenerative cartilage treatment for general repair or in the case of osteoarthritis.

Conflicts of Interest The authors have no conflicts of interest.

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Redundant Human Omentum Fat: A Leap Towards Regenerative Medicine

12

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Introduction

Mesenchymal stem cells possess a ground-breaking potential and appear to offer a wonderful opportunity, indeed a responsibility to understand important aspects of human biology involving tissue repair and regeneration [1, 2]. The ubiquitous existences of multipotent mesenchymal stem cells (MSCs) annex to be a regenerative tool rendering the replacement of worn-out cells. Researchers have averted their attention towards identification of various sources of adult mesenchymal stem cells from our own body tissues and fluids [3–11]. Despite the existence of several advantages and potentials of MSCs from several sources being investigated, bringing stem cells adaptable for regenerative medicine applications in adequate quantities at the right time is a challenge. This is with regard to the inevitable fact that the frequencies of mesenchymal stem cells and their proliferative capacities and differentiation potentials as well as phenotypical and immunomodulatory properties have been shown to vary among sources. Furthermore, cell-based therapies rely to a larger degree on the preparation of an effective dose of ex vivo expanded cells, capable of self-renewal and differentiation. The identification of physiologically relevant and ideal source of stem cells that might be more useful in clinical setting needs to be investigated to ascertain an assured quality in cellular therapy. Additionally, changing the perception, about the successful treatment of stem cells for various diseases, in the light of recent findings becomes mandatory to cure these diseases and further to broaden the potential applications of stem cells. Adult stem cell therapies are routinely used to treat diseases using umbilical cord blood stem cell transplants [12] and peripheral blood stem cell and bone marrow stem cell transplants [13–17] which are probably the most well-known therapy.

Although umbilical cord blood stem cells are a promising therapeutic determinant in regenerative medicine, their applicability is limited by the lesser frequency of MSC, HLA matching for allogenic transplantation as well as long-term storage difficulties in autologous transplantation [18]. The multitude of research on bone marrow and peripheral blood stem cell has proved them the most favourable candidate for autologous stem cell transplantation. The limitations of bone marrow and peripheral blood stem cells in terms of patient discomfort, reduction in yield and frequency of stem cells with age and body mass index, have forced the researchers to find alternate sources of stem cells [19–22]. In recent years, researches are heading towards identification of novel source of MSC from redundant tissue sources. The main factor that makes redundant tissue an attractive source of stem cell is its non-invasive nature and tissue abundance.

The major redundant tissue sources under research for stem cells are the umbilical cord tissue, cord blood, placenta, endometrial tissue, menstrual blood and subcutaneous adipose tissue. In view of the fact that most of the redundant tissue sources are pregnancy related, their applicability in

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autologous transplantation is limited to women as well as due to the aforesaid reasons to umbilical cord blood stem cells. In contradictory to the difficulties related to these sources, endometrial tissue and menstrual blood (maternity-related samples) have far wider applicability in autologous stem cell treatments. In comparison to all these above-discussed sources, yet another potent source recognised in the realm of adult stem cells similar to that of subcutaneous fat is the omentum fat that possesses far wider applicability as it is not even limited by the sex of the patient. Visceral or omentum (intra-abdominal) fat has attracted much interest than the subcutaneous fat as accumulation of excess of omentum fat is responsible for many metabolic abnormalities associated with obesity [23]. Besides, removal of omentum fat has a synergistic effect on their insulin responsiveness. This synergism arises from the higher rate of triglyceride turnover in omentum fat compared to subcutaneous depot, attributed to the decreased sensitivity towards the antilipolytic effects of insulin [24–26]. This indicates the significance of omentectomy in prevention of its deleterious effects. At the same time, although many accumulating evidences exist on the advantageous and applicability of mesenchymal stem cells derived from subcutaneous fat in therapeutics, the efficacy, feasibility and applicability of omentum fat-derived stem cells for clinical approaches remain unclear.

This chapter demonstrates an overview on the angelic and demonic side of the redundant human omentum fat. It reveals the imperativeness of omentectomy as a double-edged sword, whereby it plays an important role in reduction of pathophysiology associated with metabolic diseases as well as serves as an alternative therapeutic source for the treatment of wide range of diseases, as current research data had reported on the proliferative and multi-differentiation ability of omentum fat-derived MSC. This chapter will further confer consistency on certain unexplored aspects of the omentum fat. Overall, it is anticipated that this chapter will act as a reference for further exploration on these aspects and work towards the achievement of reduction in pathology associated with diseases as well as to create an environment where curative therapeutics can be successfully achieved using omentum fat-derived stem cell transplantation.

Omentum Fat: Angels or Demons?

White adipose tissue (WAT) present in the *omentum majus*, lying intra-abdominally between the abdominal muscles and the visceral organs, is called as omentum fat. It is also known as intra-abdominal fat, as it is located inside the abdominal cavity, packed in between organs (the stomach, liver, intestines, kidneys, etc.). It is considered to be a shock absorptive fat layer protecting the visceral organs from external impacts. Visceral or omentum (intra-abdominal) fat has attracted much interest than the subcutaneous fat as accumulation of excess of omentum fat results in production of very less fat-regulating adiponectin, leading to many of the metabolic abnormalities associated with obesity [23]. Consequently, it has become a key measurement for the definition of the metabolic syndrome [27]. These disorders include abdominal obesity, high blood pressure, high cholesterol and other risk factors associated with cardiovascular risk and type 2 diabetes risks in obesity [28, 29]. On the other hand, similar to the existence of subcutaneous fat-derived stem cells, there are evidences that omentum fat does possess putative stem cell population [30, 31]. Data reported that identification of putative stem cell population in omentum adipose tissue could represent a very useful tool to investigate, at the cellular level, the molecular mechanism and process involved in the onset of obesity and related metabolic dysfunctions. In addition, stromal cells isolated from human omentum fat were identified to retain the stem cell characteristics such as proliferative and multilineage differentiation potential inclusive of its angiogenic and regenerative potential [30–35]. Thus, just as either sides of the coin, omentum fat can be considered both as angels and demons of the body. Increasing the angelic activity and decreasing its demonic activity are of utmost importance for cure of various disorders and its regenerative therapeutics.

Omentectomy: A Double-Edged Sword

Humans have enormous quantity of omentum fat as that of subcutaneous fat, and it has been demonstrated from accumulating evidences that the omentum fat can be harvested for the isolation of stem cells, thereby providing omentum fat as a reservoir of stem cells. On the other hand, omentum adipose tissue is more closely associated with an adverse metabolic risk profile rather than subcutaneous fat. This is further supported by both animal and human studies [30, 31] demonstrating that subcutaneous adipose tissue liposuction influences BMI and overall weight, whereas only reduction of visceral fat can also substantially ameliorate metabolic parameters [36]. Thus, existing evidence supports the reduction of omentum fat tissue (omentectomy) to be beneficial in both ways and hence serving dual purpose, substantiating its imperativeness in pathophysiology as well as further stem cell-oriented clinical applications.

Pathophysiological Rationale for Omentectomy

The redundancy of excess omentum fat is reinforced by the pathophysiological complications associated with increased

omentum fat accumulation. Most of the epidemiological studies have correlated the relation between severe obesity and mortality due to increased rates of diabetes and cardiovascular and cerebrovascular diseases [37–42]. Adipose tissue is an endocrine organ which secretes peptide and protein hormones (adipokines) that regulate storage and release of energy [43]. The major adipokines of fat metabolism are adiponectin, leptin, visfatin, retinol-binding protein-4 and adipsin. Any dysregulation in the production or release of these adipokines results in metabolic disturbances leading to metabolic disorders. Adiponectin and adiponectin receptors are the major determinants which play a significant role in the aetiology of obesity-related chronic diseases [44]. High molecular weight (HMW) adiponectin is an important factor that controls the activity of the adiponectin receptors AdipoR1 and AdipoR2. AdipoR1 activates AMP kinase pathway resulting in adipogenesis and inhibition of lipolysis, whereas AdipoR2 activates peroxisome proliferator-activated receptor alpha (PPARα) pathway in liver leading to increased insulin sensitivity and decreased inflammation leading to their antidiabetic effects.

In view of the above-discussed positivity regarding adiponectin, several researches showed a reduction of adiponectin levels in patients with visceral/omentum obesity which is considered to be the reason for insulin resistance leading to type 2 diabetes and cardiovascular and coronary artery diseases [45–48]. Since adiponectin being an antidiabetic factor, its reduced production and release in obese subjects lead to chronic diabetes [49].

Leah Di Mascio and his co-workers identified that adiponectin is an essential factor for the proliferation and function of haematopoietic stem cells even in their niche [50], which may be a potential reason for the reduction in bone marrow volume in obese patients. Adiponectin has also proved its potential to induce adipocyte [51] and osteocyte differentiation [52]. The adipogenic effect of adiponectin causes the proliferation and differentiation of preadipocytes into adipocytes leading to its accumulation and obesity. There are also research outcomes suggesting the deleterious effect of omentum fat accumulation on androgen activity in female subjects. The androgen inactivation effect of omentum fat accumulation is considered to be due to the higher expression of 3α-hydroxysteroid dehydrogenase (3α-HSD) [53].

The preadipocytes of the omentum are also reported to mature into macrophages, which contribute to local immune and inflammatory responses. The formation of local immune factors such as dendritic cells in visceral fat leads to inflammatory conditions associated with Crohn's disease [54]. Further research confirms the role of visceral adiposity in the development of cardiovascular and cerebrovascular diseases. The reduced adiponectin levels associated with increased visceral adiposity cause development of inflammations and atherogenesis by the migration and maturation of monocytes and macrophages leading to their transformation into foam cells on vascular wall [55, 56]. Thus, omentectomy is emerging as a major treatment for chronic diabetes, whereby reduction in omentum fat leads to increase in production of adiponectin. In addition, omentectomy acts as a doorway towards diminutive pathophysiological deleterious effects, and it further moves a step forwards in annihilating several aforesaid debilitating disorders that occur due to accumulation of omentum fat.

Omentectomy: Promising Days in Regenerative Epoch

Harvesting of the redundant omentum fat obtained through omentectomy not only serves the aforesaid purpose but also harbours a highly heterogeneous population of cellular components that support its applicability in cellular transplantation for regenerative medicine. These heterogeneous cellular components of human omentum fat constitute the stromal vascular fraction (SVF) characterised by the abundance of preadipocytes, microvascular endothelial cells, mastocytes, fibroblasts, monocytes/macrophages, progenitor cells of bone marrow origin, leukocytes and granulocytes. The macrophage richness of omentum fat is comparatively higher than all other major adipose tissues. In fact omentum adipose tissue distribution and their cellular dynamics are determined by the proliferative and differentiation capacity of the preadipocytes. Despite the fact that SVF is a heterogeneous cell population, subsequent expansion of the SVF (preadipocyte culture) in vitro selects for a homogenous mesenchymal stem cell population that holds a greater potency for its cellular transplantation. The proliferative capacity of this adipose tissue was explored to be persistent with ageing compared to the subcutaneous adipocytes which lose its proliferative capacity with ageing [57].

These distinctive aforesaid properties of omentum fat highlight on the imperativeness of omentectomy for harvesting of stromal vascular fraction as well as homogenous mesenchymal stem cells that showcase omentum fat as a promising base for regenerative epoch. Thus, emphasising on the potential benefits of omentectomy as a double-edged sword creates a mass of attention in the midst of scientific community to research on yet another potent redundant source of stem cells, the omentum fat for its use in tissue repair and regeneration. Current research focusing on characterisation and culturing of omentum fat-derived MSC has proved its proliferative and multi-differentiation ability, guaranteeing its potential as a regenerative therapeutic source [30–33, 35]. However, there have been scanty citations with human omentum fat-derived stem cells, as the research is in its incipient stage. Hence, the efficacy and fea-

sibility of its application for clinical approaches remain unclear. This chapter had further paid attention to certain existing uncertainty by focusing towards the attributes of omentum fat-derived stem cells in view of its morphoproliferative capacity, biomarker expression and multitude differentiation potency.

Morphoproliferative Possessions

Isolation and morphological characterisation of a multipotent stromal cell population from visceral/omentum tissue have been reported to share the same morphological and electrophysiological properties of stem cells isolated from subcutaneous adipose tissue from the recent findings [30–33]. They report that omentum fat showed similar and homogenous fibroblast-like (Fig. 12.1) and similar ultrastructural organisation as detected by transmission electron microscope. In contrary, omentum fat-derived stem cells were found to differ morphologically than that of subcutaneous adipose tissue in some reports. Literature provided evidence for the omentum fat-derived MSCs being large and having more spread appearance, whereas subcutaneous MSCs have comparatively smaller size and varying morphology such as spindle-shaped neuron-like appearance [58]. Additionally, it was reported that omentum MSCs appeared to contain more fat droplets and were seldom granulated. This phenomenon was not observed in subcutaneous MSCs. It was also identified that omentum fat possesses more of blood-derived cell population than subcutaneous fat, whereas subcutaneous fat alone was identified to possess more of adipose tissue-derived cell population [30, 31]. Moreover, the comparison of replicative capacity of omentum preadipocytes to that of subcutaneous preadipocytes revealed that the proliferation capacity in subcutaneous preadipocytes was found to be superior to those of omentum cells since subcutaneous cells proliferate faster under in vitro conditions [33, 59]. On the other hand, the proliferative capacities of omentum adipose tissue were explored to be persistent with ageing compared to the subcutaneous adipocytes which lose its proliferative capacity with ageing [57].

Despite the existence of these uncertainties, omentum fat-derived mesenchymal stem cells had never lost the battle and provided evidence for it to withstand the unique characteristics of mesenchymal stem cells when competing with subcutaneous fat-derived stem cells in certain aspects. Omentum fat-derived MSC had evinced to retain its unique characteristics until extensive long-term culturing of greater than P10 as reported in literature [58]. It was confirmed by the presence of stable immunophenotypic characteristic of hMSC and the absence of any genetic transformations by stable karyotyping. However, it has been reported that omentum fat-derived stem cell exhibits the presence of CD34 and CD45 at later passages, exhibiting the presence of cells of other lineages than MSCs. A high demand on omentum fat-derived MSC applicability for clinical advances relies on the ease of their isolation, extensive capacity for in vitro expansion, their functional plasticity and retention of MSC characteristics in long-term culture condition without undergoing epigenetic changes. Although omentum fat has emerged themselves as a novel redundant source of stem cells substantiated with limited body of evidence, extensive expansion of MSC derived from omentum fat with exclusive retention of unique characteristics had not been reported yet.

In lieu of this, our group demonstrated and initiated the optimisation of culture condition for omentum fat-derived

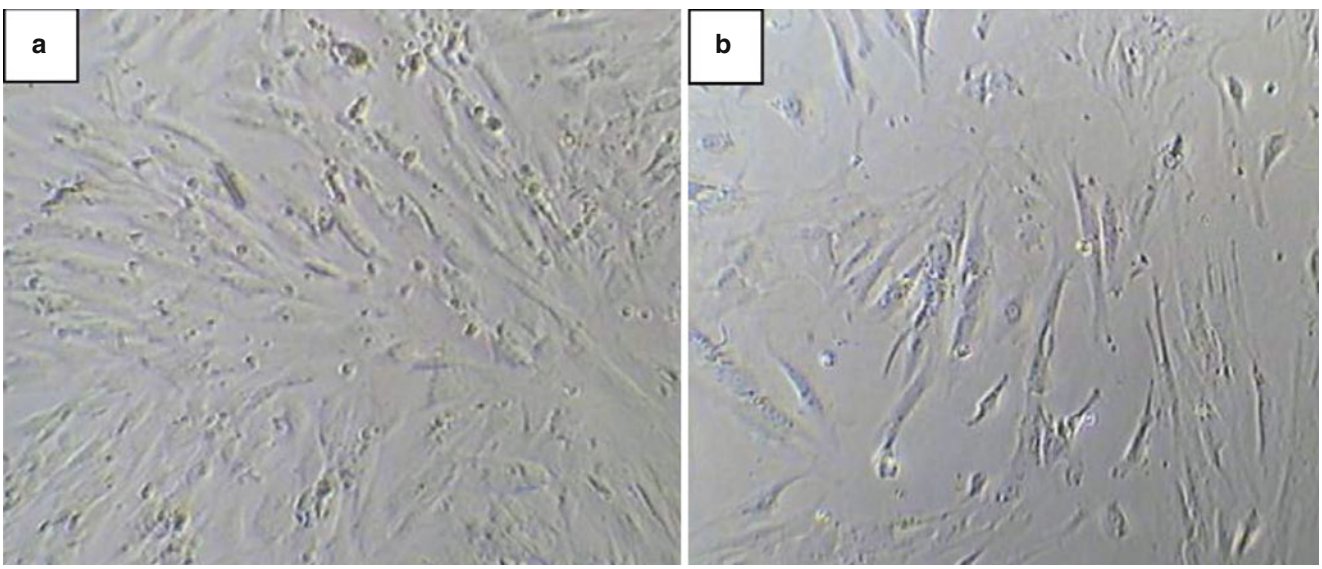

Fig. 12.1 Morphological appearance of mesenchymal stem cells derived from omentum fat

MSC by extensive culturing of these cells in different media. The study was carried out in view of its various attributes such as proliferation, phenotypic characterisation and differentiation potential, thereby exploring its imperativeness as a deserving candidature of stem cells. The study substantiated the results by all means by retaining its characteristic features under extensive culturing and hence identified as an alternative and additional redundant source of stem cells, thereby serving as an additional supportive data for enhancing its application in regenerative medicine [60].

Biomarker Expression Profile

While an extensive body of research exists pertaining to phenotypic characterisation of stem cells from bone marrow and subcutaneous adipose tissue, the phenotypic characterisation of omentum fat-derived stem cell is at its infancy. Though there is paucity on characterisation of omentum fat-derived stem cell until today, existing little evidence on immunophenotypic characterisation of omentum fat at early passage reveals the similarities on the expression of cell surface markers of omentum fat in comparison to the subcutaneous fat and bone marrow [1, 61–64]. Omentum fat was identified to express wide range of markers. The heterogeneous stromal vascular fraction isolated from omentum fat was found to express the cell surface markers specific for haematopoietic stem cells, mesenchymal stem cells, cell adhesion molecules, endothelial cells and other non-stem cell population as well (Table 12.1 and Fig. 12.2).

Although omentum fat has evinced themselves as a potent source of stem cells in certain attributes, there is no unique biomarker that can reliably be identified specific to omentum fat-derived stem cells. Identification of these unique markers potentially serves several purposes both in terms of identification of the cellular and molecular mechanism of the diseased status and specific markers of MSC, by which taking a step forwards from bench to bedside. From the expression profile of the markers identified so far in current studies, it was presumed that, under culture condition, the omentum fat expresses remarkable expressions of the MSC markers (CD90, CD105 and CD73) as reported by ISCT [65] and also expresses various cell adhesion molecules such as CD29, CD44, CD31, CD106, CD54, CD49d and CD166 favouring the migratory and regenerative potentialities of omentum fat-derived MSC. In addition, the cell surface markers such as CD34, CD31, CD45, CD133 and HLA-II were found to be decreasing/negative in its expression [30, 32, 33, 35, 58] (Table 12.1). Apart from the expression of the routine cell surface markers, omentum fat also possesses the expression similar to the expression of embryonic stem cell marker such as Oct 4, Sox 2, Nanog and so on. This explains not only its multipotent nature but also pluripotent property of omentum fat, thereby enhancing its applicability (Table 12.1).

Table 12.1 Cell surface marker expression profile of freshly isolated SVF versus cultured MSC derived from omentum fat

Markers	SVF	OF-MSC
CD45	+	–
CD31	+	–
CD34	+	+
CD105	+	+
CD14	+	–
CD44	+	+
CD29	+	+
HLA-I	+	+
HLA-II	+	–
CD106	+	–
CD146	ND	–
CD90	+	+
CD13	ND	+
CD133	+	–
CD73	+	+
CD49d	–	+
CD166	–	+
CD54	+	+
ABCG2	–	+
ALDH	+	+
CD117	+	+
LIF	ND	+
Keratin 18	ND	+
SOX2	ND	+
Nanog	ND	+
Oct-4	ND	+
SSEA-1	ND	+
SDF-1α	ND	+
CXCR4	ND	+
WT-1	ND	+
VEGF	ND	+

Refs. [30, 32, 33, 35, 58]
SVF stromal vascular fraction, *MSC* mesenchymal stem cells, *OF* omentum fat

The marker positivity explained involves its expression pattern in early passage condition. However, retention of its marker positivity for longer culture period substantiates the identification of unique marker specific to MSC derived from omentum fat. Thus, results obtained from our study emphasise the fact that all media articulate almost similar expressions of all the markers except slight variations. Two attributes clearly became evident regarding the withholding capacity of the cell surface marker expression until extensive culturing. Firstly, as omentum fat does not lose its phenotypic characteristics in prolonged culture condition and maintain the similar phenotypic expression throughout, it deserves to be an efficient alternative source of curative therapeutics. Secondly, it is evident that five media inclusive of

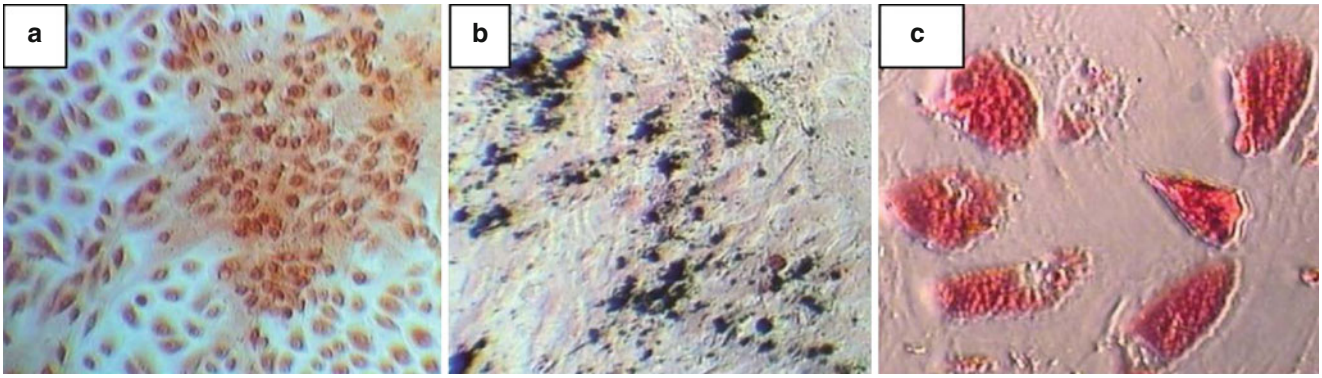

Fig. 12.2 Confirmation of osteogenesis and adipogenesis: (**a**) alizarin red staining and (**b**) von Kossa staining of osteoblast calcium deposits; (**c**) oil red O staining of lipid vacuoles in adipocytes

lower and higher glucose concentrations do not affect the phenotypic expression profile of these cells in longer passages [unpublished data]. Hence, it can be concluded that omentum fat can be regarded as an efficient alternative source of curative therapeutics with regard to the cell surface antigenic expression profile. However, other attributes such as its multitude differentiation ability are mandatory to confirm the potency of omentum fat-derived stem cells.

Multitude Differentiation

Adipose-derived stem cells have been proved to be capable of multilineage differentiation [66, 67]. As stem cells from adipose tissue have their origin in the mesoderm, it is no longer surprising that these cells can undergo different mesenchymal lineage conversions. Accordingly, omentum fat-derived stem cells have a higher capacity to differentiate into both osteogenic and adipogenic lineages. It was noted that, however, omentum fat differentiation into adipocytes is inferior to that of subcutaneous fat [33, 68]. When maintained in appropriate inductive medium, omentum fat-derived stem cell cultures displayed in vitro multipotency and were proved differentiation towards specific lineages. Omentum fat-derived stem cells were able to differentiate into mesodermal lineages of adipogenic, osteogenic and chondrogenic lineages. The differentiation ability of omentum fat-derived stem cells was first identified in rat in the year 2003 by SS Tholpady and his co-workers [31]. They reported that the visceral fat of rats contains MSCs with multilineage differentiation ability like subcutaneous fat despite the well-documented difference in the metabolic and biochemical properties among anatomically distinct depots of fat.

Later, multilineage differentiation potency of human omentum fat-derived stem cells as compared to human subcutaneous fat became evident [33, 68]. Additionally, the property of lipolysis of both differentiated adipocytes derived from omentum fat and subcutaneous fat was evaluated. Besides adipogenic differentiation, osteogenic differentiation of human omentum fat-derived stem cells was also identified. The presence of mineralised calcium nodules was confirmed in vitro using different staining methods such as alizarin red and von Kossa. It was also verified using the presence of genes for the osteoblast. It was identified that the omentum fat-derived stem cells were inferior in osteogenic differentiation when compared to subcutaneous fat. It is apparent that there are scanty citations of differentiation potential of omentum fat, thus creating an uncertainty in the efficacy of the differentiation potential of omentum fat.

Transdifferentiation

It was not surprising that the mesenchymal stem cell being mesodermal in origin differentiated into mesodermal lineages. To possess the attributes of mesenchymal stem cells, it is noteworthy that the omentum fat-derived MSC should undergo transdifferentiation. The ability of its transdifferentiation capacity will provide evidence for its plasticity and regenerative potency of omentum fat-derived stem cells. Although the existence of MSC is demonstrated with its multilineage mesodermal differentiation, not much is studied with respect to its transdifferentiation ability. Tholpady and his co-workers had reported that the rat omentum fat-derived stem cells could differentiate not only into the mesodermal lineages of osteocyte, adipocyte and chondrocyte but also undergo neuronal differentiation [31]. This phenomenon of differentiation of omentum fat-derived stem cells into neuronal lineage was also studied by Paul Kingham [69]. However, until recently, transdifferentiation of omentum fat-derived stem cells to other lineages was not demonstrated yet. It is of paramount importance that omentum fat-derived MSC possesses the ability to transdifferentiate in multitude of lineages before it has been considered efficient in regenerative medicine. However, regenerative potency of the omentum

fat-derived mesenchymal stem cells has also been elucidated by Rahim and his co-workers [34]. It was described that the freshly isolated whole cell population of omentum fat can regenerate sciatic nerves when injected into a mice model. They concluded that it may consider clinically as a translatable route towards new methods to enhance peripheral nerve repair without the limitations of BMSCs in clinical application. This reveals the fact that omentum fat can be considered further for study in the emerging field of regenerative medicine and surgery.

As omentum fat is associated with the metabolic disorders such as diabetes, it is noteworthy that besides being a potent source of MSC, removal of omentum fat from diabetic patients for the treatment of diabetes has a synergistic effect on their insulin responsiveness. This synergism arises from the higher rate of triglyceride turnover in OF compared to SF depot, attributed to the decreased sensitivity towards the antilipolytic effects of insulin [30, 31]. Hence, the wonder-working potential of the transdifferentiation of omentum fat-derived MSC into pancreatic islet-like clusters might bring a new era in the field of diabetology. Our group identified that the preliminary data on transdifferentiation of omentum fat-derived stem cells into pancreatic islet-like cluster revealed a positive expression of certain specific genes and morphological islet-like cluster [60].

Concluding Remarks

Overall, the wealth of knowledge on the biological function of white adipose tissue, especially omentum fat, has evinced them as an endocrine organ that is strongly associated with metabolic complications of obesity leading to debilitating diseases such as diabetes and cardiovascular diseases. On the other hand, new insight on omentum fat emerged when studies reported the existence of stem cells and emphasised its pivotal role in tissue repair and regeneration, thereby paving way for the researchers to identify both the angelic and demonic side of omentum fat. This further created an environment for the researchers to explore the imperativeness of double-edged sword of omentectomy, whereby working towards annihilating several aforesaid debilitating disorders that occur due to accumulation of omentum fat, on one side, and achieving curative therapeutics using omentum fat-derived stem cell transplantation, on the other side.

Furthermore, the perception of the far wider potential of mesenchymal stem cells derived from omentum fat has led to considerable excitement and a leap towards potential therapeutic applications in regenerative medicine and tissue engineering. Nevertheless, the incipient stage of the research on omentum fat-derived stem cells and its potential application becomes a major stumbling block for its advancement in regenerative medicine. Hence, a lot needs to be explored, inclusive of its proliferative and multitude differentiation ability, to rationale omentum fat as a successful redundant source of stem cells.

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Human Adipose-Derived Stem Cells (ASC): Their Efficacy in Clinical Applications

13

Michelle B. Locke and Vaughan J. Feisst

Abbreviations

Ag	Antigen
AITP	Autoimmune thrombocytopenic purpura
ASAPS	American Society of Aesthetic Plastic Surgeons
ASC	Adipose-derived stem cells
ASPS	American Society of Plastic Surgeons
BCT	Breast-conserving therapy
BMSC	Bone marrow stem cells
CAL	Cell-assisted lipotransfer
CHD	Coronary heart disease
CT	Computed tomography
ESC	Embryonic stem cell
FI	Fat injection
GVHD	Graft-versus-host disease
HCN	Hyperpolarization-activated cyclic nucleotide
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
IFATS	International Fat Applied Technology Society now known as the International Federation for Adipose Therapeutics and Science
ISCT	International Society of Cellular Therapy
IV	Intravenous
MS	Multiple sclerosis
MSC	Mesenchymal stromal/stem cell
NIH	National Institutes of Health
OA	Osteoarthritis
PLGA	Poly(lactic-co-glycolic) acid
POH	Progressive osseous heteroplasia
RA	Rheumatoid arthritis
RBC	Red blood cells
SC	Schwann cell
SLE	Systemic lupus erythematosus
SVF	Stromal vascular fraction
TGFβ	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

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Introduction

Mesenchymal stem cells (MSC) have been used for therapeutic purposes for many years. Multipotent cells in adipose tissue were postulated to exist by Kaplan and colleagues during their investigations into a disease called progressive osseous heteroplasia (POH) [1]. In this disease, bone forms in atypical locations, such as in subcutaneous fat or in muscle [2]. Interest in multilineage cells from adipose tissue gained momentum after 2001, when Zuk and her colleagues published their sentinel paper showing differentiation of stromal-type cells from adipose tissue along adipogenic, chondrogenic and osteogenic lineages [3]. Such has been the explosion of interest in the topic, a Medline search with "adipose" and "stem" and "cell" as the key words returns nearly 3,000 articles published since this time, compared with only 300 in the 30 years prior to this. The cells isolated from adipose tissue have been given various names, including adipose stem cells, adipose-derived stem cells, adipose-derived stromal cells, among others. A consensus statement was published following the International Fat Applied Technology Society (IFATS, now known as the International Federation for Adipose Therapeutics and Science) 2nd international meeting in 2004 [4], concluding that they should be referred to as adipose-derived stem/stromal cells (ASC) to promote consistency across research group; hence, this is the terminology used in this article.

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As adipose tissue is of mesodermal origin, ASC are a form of adult MSC. The minimum criteria for defining a multipotent mesenchymal stromal cell have been proposed by the International Society of Cellular Therapy (ISCT) to be:

1. Adherence to plastic
2. Specific surface antigen (Ag) expression
3. Differentiation potential into osteoblasts, adipocytes and chondroblasts [5]

These criteria are met for human ASC, among several other mesenchymal cell types, such as bone marrow stem cells (BMSC) and umbilical cord blood stem cells. However, while there is good evidence that ASC can differentiate along mesenchymal lineages [3, 6–8], ASC have also spurred interest due to early reports of potential transdifferentiation of the cells along nontraditional lineages, such as neuronal and epidermal cells [9–11]. As with many MSC, ASC are relatively immunoprivileged [12]. They do not express HLA class II antigens and are able to downregulate the innate immune system [13]. Therefore, their use in immune modulation, such as for the treatment of autoimmune disorders or transplant rejection, is another large area of interest [14–16].

ASC are found in the perivascular region of white adipose tissue, which includes subcutaneous fat deposits [17, 18]. These are most readily accessed surgically by liposuction (also known as liposculpture or lipoplasty). According to the latest statistics from the American Society of Aesthetic Plastic Surgeons (ASAPS), liposuction was the most popular cosmetic surgery procedure in the United States in 2011, with 325,332 procedures being performed. This represented a nearly 13 % increase in the procedure compared with the year before and an increase of over 80 % on the 176,863 liposuction procedures performed in 1997 [19]. As liposuction has been shown over many years to be a popular, safe procedure with a low complication rate [20–22], the resulting lipoaspirate seems to be an ideal source of ASC for clinical use [23]. Many protocols for isolation of ASC from adipose tissue have been published and most are substantially similar [3, 24, 25]. In short, the lipoaspirate is washed and red blood cells (RBC) are removed or lysed. The washed adipose tissue is then digested with collagenase for 30 or more minutes. The digested tissue is then filtered to remove any residual tissue and then centrifuged. The resulting cell pellet is known as the stromal vascular fraction (SVF). The SVF contains many different cell types, of which ASC are one [23, 26].

Given the huge interest in ASC and the massive variety of their potential uses, it is easy to forget that research into ASC began a mere decade ago. It is important to objectively assess what has been achieved so far and review the current level of evidence of the clinical efficacy of ASC. This chapter will therefore focus on analysis of the completed and ongoing clinical trials of human ASC. It will cover the major areas of research which have progressed as far as relevant in vivo experiments, human case reports and phase one and two trials. Their current and potential therapeutic use in humans will be discussed.

Clinical Applications

As alluded to above, there are a myriad of potential clinical applications of ASC. These include reconstruction of soft tissue defects, bone and cartilaginous defects, skeletal and cardiac muscle regeneration, wound healing and skin repair, nerve and solid organ regeneration as well as immunomodulation applications.

Adipose Tissue Reconstruction

Of all of the potential clinical applications of ASC, the most likely to be efficacious is the use of ASC to provide adipose tissue for the reconstruction of soft tissue defects. Subsequently, there is extensive literature on the clinical efficacy of ASC in soft tissue regeneration or reconstruction. Fat injection (FI), where the whole of the aspirated fat is reinjected without any processing to separate or concentrate the ASC, has been performed since 1893 for reconstructive purposes [27]. The 2011 statistics from the American Society of Aesthetic Plastic Surgeons (ASAPS) show that FI is commonly performed by their members, with 69,877 cases reported for the year 2011. This made it the ninth most common surgical procedure performed, outranking forehead (10th), thigh (14th) and arm lifts (12th) in popularity [19]. The most commonly discussed clinical use of FIs today is breast soft tissue reconstruction. In the breast, FIs are performed for both purely cosmetic purposes (breast augmentation) [28, 29] and reconstructive purposes in women with congenital abnormalities or breast cancer, following partial or total mastectomy [29, 30–32]. Dr. Delay and colleagues in Lyon, France, have documented their experience with 880 breast FI procedures, of which 734 (83.4 %) were for reconstruction. They document one major complication (pneumothorax) but otherwise low, minor complication rate. They suggest that the procedure is efficacious, with long-term follow-up showing a resorption rate of 30–40 % [32]. Similarly, Dr. Rietjen and colleagues in Bangkok, Thailand, performed 194 FI procedures on 158 patients and report a low complication rate (3.6 %) [31]. They state that it is difficult to predict the percentage of fat graft survival, but only 16.8 % of patients in their series required a second FI procedure, implying good efficacy of the initial operation. Dr. Petit's team in Milan, Italy, treated 321 patients from 1997 to 2008 with FI following partial mastectomies and report no increase in breast cancer recurrence during their follow-up period.

While there are undoubtedly ASC within the transplanted fat, FIs rely mostly on volume from the transplanted adipose tissue rather than ASC for the clinical results. Some surgeons find the long-term survival of such fat grafts to be unpredictable [33]. The best methods to harvest, process and reinject fat to improve viability have been debated for many years [34–36]. Recently, to improve the "take" of the FI, the process of cell-assisted lipotransfer (CAL) is being suggested

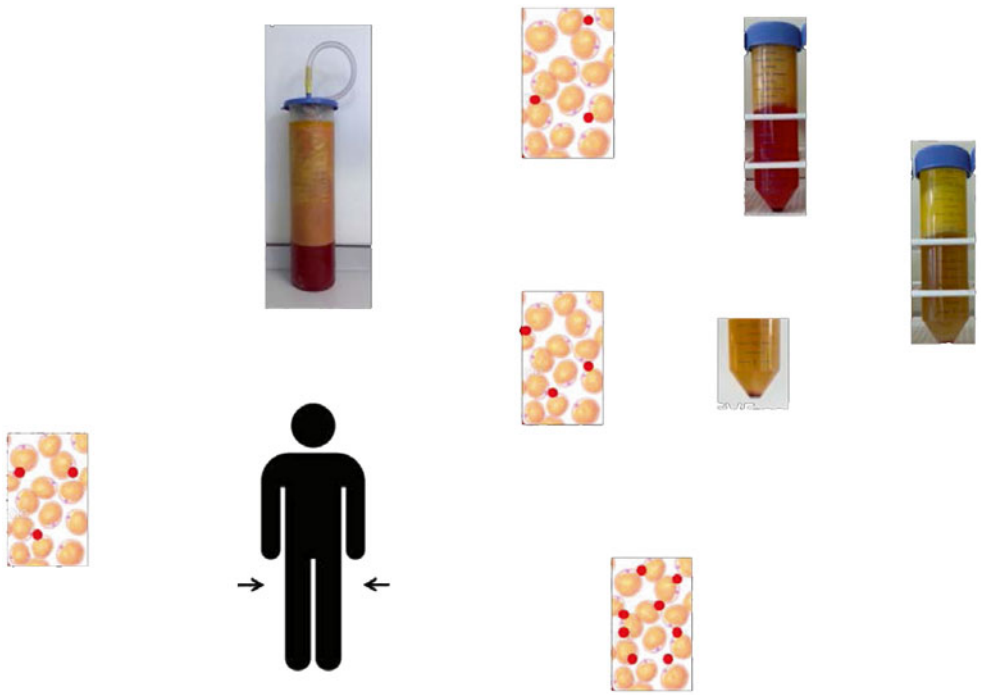

Fig. 13.1 Traditional fat injections (*FI*) compared with cell-assisted lipotransfer (*CAL*)

[37, 38] (Fig. 13.1). This is a process by which the lipoaspirate acts as the scaffold for the injection of ASC. The lipoaspirate is divided in two portions after removal. One portion is processed by standardised techniques to isolate the SVF or ASC. These cells are then mixed with the remaining, unprocessed lipoaspirate, and this enriched lipograft is used for reinjection. Yoshimura and colleagues have published their experience with a clinical trial of CAL which started in 2003. At the time of reporting, 307 patients had undergone CAL fat grafting, the majority of these (269 patients, 87.6 %) to the breast. Their 3D measurements enabling volumetric evaluation of the breast mound post-operatively showed that the graft take averaged between 40 and 80 % [39]. This is well within the range of expected long-term survival of grafts following traditional, non-enriched FI, which is reported to be anywhere between 10 and 100 % [33, 40–42, 43]. Given the great variability reported, it seems reasonable to assume that the actual efficacy of the procedure probably lies somewhere in the middle of this range. As the patients in Yoshimura's study were not compared to a cohort of patients undergoing traditional FI, it is difficult to know the role that ASC played in the clinical results. To try to address this issue, Dr. Sterodimas and colleagues from Brazil recently published the outcome of a comparative trial of the two techniques [44]. There were only ten patients in each arm of the study, but the results showed that the patients treated with a form of CAL only required one treatment for a satisfactory correction of their soft tissue defects while the control arm (traditional FI) required a greater number of operations to achieve a satisfactory correction. This implies that the survival of the fat grafts may be better with CAL. However, good outcomes were achieved in both groups, and there was no statistically significant difference between the two groups in terms of patient satisfaction at 18 months [44]. Several biotechnology companies are developing automated systems for the processing of lipoaspirate in the operating room. A multicentre, post-marketing trial of the use of Cytori Therapeutics Inc.'s Celution® system (RESTORE-2 trial) followed 71 patients prospectively enrolled for reconstruction of lumpectomy defects with CAL [45]. Forty-seven had one procedure and 24 needed two procedures for treatment. Sixty-seven of the patients completed follow-up for 1 year. Patient satisfaction at 12 months was 75 %. The authors concluded that improved breast contour was seen on MRI sequencing in 54 of 65 patients imaged. Overall, these results are subjective, and the duration of follow-up is too short to allow any meaningful comment on the long-term outcome. However, it seems reasonable to assume that CAL may be an efficacious use of ASC for soft tissue reconstructive purposes, to improve the survival of the transplanted fat grafts.

While fat grafting to the breast may be an effective treatment, it is not without controversy. In 1987, the American Society of Plastic Surgeons (ASPS) issued a position paper unanimously deploring the use of autologous fat injection in breast augmentation [46]. This was based on the possibility of post-operative scarring causing micro-calcifications which may hamper the detection of early breast cancer. Subsequently, only a few surgeons in the United States have admitted that they perform this procedure [29, 47]. However, the popularity of FI to the breast has increased in Europe and Asia over this time. In May 2011, a joint position statement was issued by both the ASAPS and ASPS addressing stem cells and fat grafting [48]. This concluded that there is encouraging data from laboratory studies to suggest that the use of ASC was a

promising field but that there is very little good clinical evidence to support many of the advertised claims. The responsibility has now been largely turned back onto the physician to practice safely and to collect and report outcome and safety data. It is likely that this change in position will result in a rapid growth in the number of FI or CAL procedures performed in the United States over the next decade, as they catch up with current trends in Europe and Asia.

Wound Healing and Skin Engineering

ASC have great potential for use in human wound healing and skin engineering because of their ability to promote neovascularisation and inhibit inflammatory responses. Some of the earliest phase I and II clinical trials of ASC were in the treatment of chronic fistulae in Crohn's disease by a group in Madrid [49–51]. Initially, they had success healing a single rectovaginal fistula with autologous ASC in 2003 [49]. This was followed up with a small phase I trial enrolling four patients with nine fistulae, all of which had failed traditional medical and surgical treatment at least twice [50]. ASC were harvested, cultured for up to a month, and then injected directly into the fistula sites. Six of the eight followed up regularly were healed at 8 weeks (75 %). The authors have followed this up with a phase II trial [51]. While these results are very encouraging, the exact mechanism of action of the ASC in healing is as yet unclear. These could include direct differentiation of ASC into the epithelium or support for angiogenesis of the local tissue or other paracrine effects from the release of cytokines and growth factors to the area. Regardless of the mechanism, these reports have fuelled clinical research in this area.

To this end, FIs have been used to aid healing of radiation-induced ulcers and even osteoradionecrosis [52, 53]. While the healing process was attributed to ASC in the case discussed by Rigotti and colleagues, no actual evidence for this was given in the paper. ASC have also been investigated in an in vitro model of laryngeal tissue engineering, showing potential differentiation into the epithelium on a fibrin scaffold [9]. Epithelial differentiation may be one of the many useful facets of ASC in wound healing. However, the "holy grail" of wound healing is to be able to generate full-thickness skin. Currently, there are no human clinical trials exploring the use of ASC in skin engineering, which is still in the experimental stages of development and has so far been predominantly limited to in vitro investigations. When engineering human skin, it is possible to go directly from in vitro investigations to human clinical trials, as long as the product can be shown to have critical characteristics similar to native human skin. Engineering full-thickness human skin for clinical use generally involves combining fibroblasts and keratinocytes with a dermal substitute in vitro, culturing for a number of days and followed by grafting onto a patient's wound bed. Fibroblasts and keratinocytes are isolated from either autologous or allogeneic sources, expanded in culture until the required cell numbers are achieved and then seeded onto a dermal substitute, which acts as a scaffold on which skin can form. Important features required for effective skin formation are fibroblast migration into the dermal substitute and the keratinocytes forming a stratified epidermis, including a stratum corneum to provide the skin with barrier function. Once this is achieved, the engineered skin is ready to be grafted onto a patient's wound bed.

A number of engineered skin products have been developed for clinical applications such as treating burn wounds, laryngeal scarring and chronic diabetic ulcers, all with advantages and limitations [54]. A significant clinical problem for skin engineering is vascularisation [54]. When engineered skin is grafted onto the wound bed of a patient, it does not have any vasculature. Neovascularisation of a full-thickness engineered skin graft must derive from the wound bed in an efficient and timely manner to ensure survival of a graft. ASC have been compared to dermal fibroblasts and have been shown to be very similar with respect to cell morphology and cell surface phenotype [55]. A significant difference between the two cell types is the enhanced ability of ASC to secrete pro-angiogenic factors which can promote neovascularisation [55]. Another difference is the ability of ASC to directly differentiate into an endothelial cell lineage [56]. Both of these mechanisms could provide a significant benefit over the traditional use of fibroblasts for full-thickness skin engineering. Along with these characteristics, the relative ease of access to large numbers of cells and the anti-inflammatory effects of ASC (discussed later in this chapter) contribute to their attractiveness for use in skin engineering [57].

A number of full thickness skin engineering methods substituting ASC for dermal fibroblasts have been trialled. One method grew sheets of human dermal fibroblasts and ASC separately and subsequently seeded the cell sheets with human keratinocytes to form a full-thickness skin graft [58]. The engineered skin grafts containing either ASC or dermal fibroblasts were grafted onto nude mice and analysed 21 days later. Histochemical analysis of the grafts demonstrated that both the ASC and dermal fibroblast containing skin grafts were able to form a stratified epithelium which included a stratum corneum [58]. Other investigations have used dermal substitutes to engineer full-thickness skin grafts. Commercially available collagen-based dermal substitutes such as Matriderm® are commonly used in skin engineering. Using this scaffold, a full-thickness skin graft with human ASC and keratinocytes was engineered, which demonstrated that ASC were able to migrate into the dermal substitute, similar to dermal fibroblasts [59]. Another investigation using collagen as a dermal substitute with human ASC, dermal fibroblasts and keratinocytes demonstrated that using ASC produced full-thickness skin with a stratified epidermis of equal quality to dermal fibroblasts [60]. This group also demonstrated that if ASC and dermal fibroblasts were combined in the dermal substitute, the epidermis that resulted was thicker than with either cell type alone [60]. These

investigations have shown that ASC are able to substitute for dermal fibroblasts in full-thickness engineered human skin.

Auxenfans and colleagues have partially addressed the question of whether ASC have any benefit to neovascularisation of full-thickness engineered human skin. Using a collagen-GAG-chitosan dermal substitute, full-thickness skin was engineered with ASC, dermal fibroblasts, or ASC that had been differentiated into endothelial cell lineage [61]. In all three cases, a stratified epidermis was formed, but only in the skin engineered with ASC differentiated into an endothelial cell lineage was there evidence of vascularisation of the dermal substitute. This investigation demonstrated that ASC differentiated into an endothelial lineage can be used to enhance vascularisation of engineered human skin.

While no clinical trials in this field have yet been published, the research discussed above shows that ASC may have benefits to use over fibroblasts in full-thickness engineered human skin (Fig. 13.2). It may also be possible to

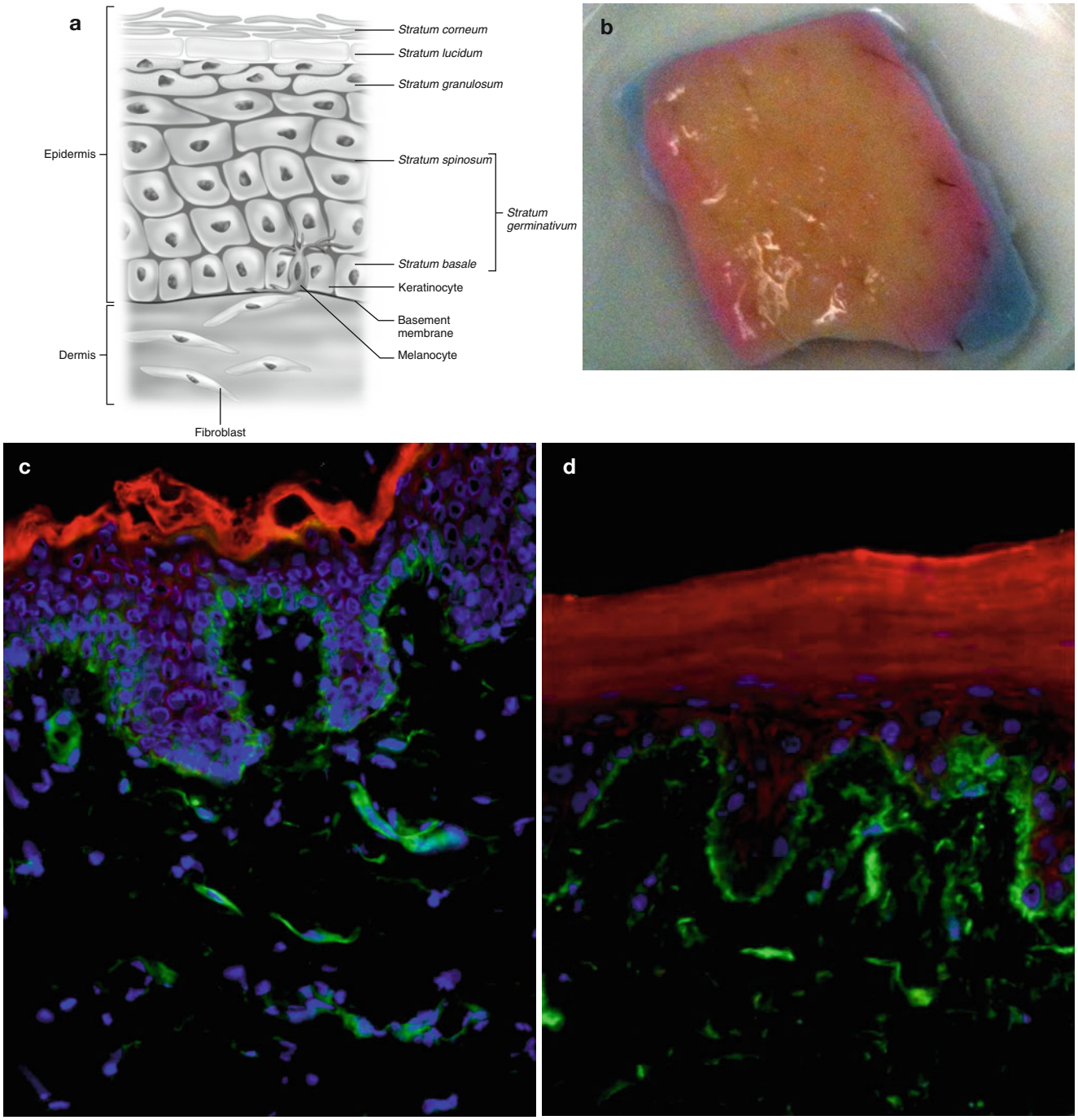

Fig. 13.2 Full-thickness engineered human skin. Cartoon depicting the layers of human skin (**a**). An image of human skin engineered on acellular, de-epithelialised dermis stained with a viability dye to show dead tissue in *blue*, live tissue in *pink* and epidermis in *yellow* (**b**). Transverse sections of normal human skin (**c**), and human skin engineered on acellular, de-epithelialised dermis (**d**) stained for pan cytokeratin (*red*), integrin β1 (*green*), cell nuclei (*blue*)

use a banked, allogenic ASC cell line due to their immunoprivileged status. The most significant advantage of using a banked ASC cell line over autologous dermal fibroblasts is that expanding cells from a patient biopsy (which is a time-consuming process) would not be required; allogeneic ASC would be immediately available. Currently, it is not clear whether ASC provide any benefit to neovascularisation of the engineered skin, either by secreting paracrine factors to promote neovascularisation from the wound bed or by themselves differentiating into an endothelial cell lineage and filling the vascular niche. Under normal in vitro conditions in engineered skin, there is no stimulus for ASC to differentiate into an endothelial cell lineage. Therefore, in order to determine whether ASC are able to promote neovascularisation of the engineered skin, a source of endothelial or endothelial progenitor cells will be required, either supplied in the engineered skin or derived from the wound bed. However, research is progressing rapidly in this field, and it seems likely that ASC will be efficacious in clinical skin reconstruction.

Skeletal Reconstruction

Degenerative joint disease (osteoarthritis, OA) affects 13.9 % of adults aged 25 and older and 33.6 % of those over 65 years [62]. Increases in disease burden are expected due to aging populations. Also, there is a strong clinical need to generate bone for the repair of large osseous defects which would traditionally require vascularized bone grafts for reconstruction [63]. Add to this the disease burden of bony non- or malunions, and it becomes clear that alternative treatment options for bone and joint reconstruction would be highly valuable clinically and potentially relevant to millions of older adults. Considering that bone and cartilage are two of the mesenchymal lineages into which ASC were first identified as being able to differentiate [3, 7], it is somewhat surprising that clinical use of ASC for skeletal reconstruction is not further advanced. Reasons for this lag are numerous and include inefficient differentiation protocols, inability to produce elastic or hyaline cartilage types and the wide variety of reconstructive methods used by researchers.

One of the earliest papers looking at ASC for bony wound healing came from Cowan and colleagues in 2004. ASC seeded onto PLGA scaffolds were used to repair critical-sized cranial defects in mice [64]. Systemic transplantation of human ASC into mice has recently been shown to increase the number and function of osteoblasts and osteoclasts [65], implying that bone repair could be improved by this method. Also in 2004, the first case report was published showing healing of a critical-sized cranial defect in a 7-year-old girl, attributed to ASC [66]. However, in this case the entire SVF was used and mixed with a more traditional cancellous bone graft, fibrin glue and an absorbable scaffold. While a promising start, this combination made interpretation of the role of ASC in the eventual healing more challenging. More recently, a small phase I trial was published which included four patients with large cranial defects who were treated with a combination of ASC mixed with b-tricalcium phosphate granules (and resorbable mesh in two patients) [67]. Improved ossification was seen in all cases on follow-up computed tomography (CT) scanning.

This latter study is typical of current research into bone- or cartilage-based skeletal reconstruction. Most researchers are assessing the role of adding ASC or other MSC into a combination treatment which also includes more traditional treatment modalities such as grafts, glues and scaffolds, sometimes including supplementation with differentiation factors [68–70]. While these are promising initial reports, many of these early studies have been performed in small animal models and have identified bone formation by non-specific methods such as von Kassa staining [68] or cartilage formation by type II collagen expression [69]. To confirm that the differentiated cells (and corresponding tissue formed) closely approximates native tissue, it needs to be compared to native tissue by analysing tissue-specific gene expression patterns [63]. To date, it seems that forms of fibrocartilage have been generated from ASC but not elastic or hyaline cartilage, as would be necessary for joint reconstruction [71]. Researchers have identified that larger doses of growth factors and shear stressing assist with chondrogenesis in ASC [72, 73]. Providing the most ideal physical and chemical microenvironment may be the key to making clinical progress in this area.

Organ Regeneration

Human ASC are being investigated for their potential utility in organ regeneration or repair. Preliminary in vitro analysis of ASC has shown that they can differentiate into cells which acquire hepatocyte-like characteristics under appropriate culture conditions and that transplantation of ASC can improve hepatic function [74]. While the mechanisms of these functional changes have not been fully elucidated, these results imply that ASC may be useful in liver or hepatocyte regeneration. Injection of human ASC into the tail vein of mice has recently been shown to lead to multiorgan engraftment of the cells [75]. When the mice were sacrificed 2 months following injection of labelled ASC, tissue samples showed the presence of human-derived cells in the bronchus of the lungs, gastrointestinal tract and liver. In the liver, human-derived cells occupied a significant proportion (6.9 %) of the tissue specimens. The hepatocytes were seen to be actively functioning by the presence of human albumin in the mouse serum. Interestingly, no human cells were found in

many other tissue types, including skeletal and cardiac muscle, the lungs, kidneys or skin. However, this result supports the clinical use of ASC in hepatic regeneration, which is currently the focus of at least three clinical trials registered with the US National Institutes of Health (US NIH).

Another aspect of organogenesis where ASC may play a clinical role is in urinary bladder reconstruction or the treatment of urinary stress incontinence. Jack and colleagues have successfully used ASC on a poly(lactic-co-glycolic) acid (PLGA) scaffold to reconstruct half a bladder wall in a nude mouse model [76]. Meanwhile, Yamamoto and colleagues in Japan have treated two patients with moderate to severe stress incontinence following radical prostatectomy with CAL injections, which were placed transurethrally into the submucous space of the urethra [77]. They report a very short follow-up of only 12 weeks but state that improvements were seen within 2 weeks. However, it is still debatable whether the primary clinical effect comes from the volume effect of the injected fat or from the ASC themselves. Another group have previously shown that there was no difference between fat and placebo (saline) injections used for the treatment of female urinary incontinence at 3 months follow-up and suggested that the fat may not be able to survive at the injection site [78].

While in vitro research and early clinical trials are ongoing in this field, it seems unlikely that the use of ASC for clinical organ regeneration will be a reality in the near future.

Cardiac Regeneration and Repair

Analysis of American heart disease statistics shows that over eight million Americans suffer from myocardial infarction (MI) every year and nearly 8 % of American adults over the age of 20 years are estimated to have some form of coronary heart disease (CHD) [79]. CHD leads to an average of one death per minute in the United States [80]. Following coronary artery blockage, there is distal ischaemia and subsequent death of the cardiomyocytes. The heart is considered to be an organ with very limited capacity for self-renewal and repair, with an inability to adequately replace damaged myocardium [81]. Given the massive societal cost of cardiac disease, ASC and other forms of MSC are being assessed for their potential as a regenerative therapy. The presumed mechanisms are both direct engraftment of the transplanted ASC and beneficial paracrine activity, with induction of angiogenesis and regulation of the inflammatory responses by vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ) and hepatocyte growth factor (HGF) [82]. As far back as 2004, Planat-Benard and colleagues published their identification of a miniscule subpopulation (0.02–0.07 %) of cardiomyocyte-like cells in murine SVF which exhibited contractile ability at day 24 following primary culture [83]. They found these cells to be positive for several cardiac-specific genes, including GATA-4 and Nkx2.5. At that time, this differentiation pathway was considered highly unlikely, but the publication fuelled in vitro then in vivo research in the field, which is continuing today. Clinical use of ASC in the treatment of chronic cardiac disease or acute MI in humans is only just beginning in early phase trials.

In 2007, Song and colleagues from the United States showed a similar, tiny (0.005–0.07 %) subpopulation of spontaneously beating cells in human SVF [84]. This occurred when a high level of VEGF was present in the surrounding media. They tested for cardiac troponin T and myosin light chain 2v and found these became positive after 12 days of cultivation. Cell to cell interaction has also been identified as a key induction requirement for cardiomyocyte differentiation in human ASC [85]. These results were optimised by coculture with contractive cardiomyocytes, suggesting an important role for paracrine signalling in the differentiation process. Recent research has also identified that both human ASC and BMSC have the ability to form sinus node-like cells [86]. ASC were felt to have a stronger proliferative capacity and express higher levels of hyperpolarization-activated cyclic nucleotide-gated (HCN) genes, which code for ion-gated HCN channels essential for generating rhythmic activity within clusters of cardiac cells. This means that ASC may potentially be useful in sinus bradycardia disease.

Translation of laboratory-based research into human clinical trials is based on animal models which have shown improvement in the recovery of cardiac function after experimentally induced cardiac injuries with direct injection of ASC. ASC have been introduced intravenously and via intramyocardial injection, with fresh and cultured cells [87]. All except one [88] have shown a beneficial effect on cardiac function [89–91]. Few results from human clinical trials have yet been published. Friis and colleagues in Denmark have published a safety and feasibility study looking at intramyocardial injection of cultured BMSC in patients with stable CAD [92]. They treated 31 patients and concluded that the treatment was safe and resulted in significant improvements in left ventricular function and exercise tolerance. They are following this up with a randomised, double-blind, placebo-controlled trial using ASC instead of BMSC for the treatment of CAD [93]. The US NIH clinical trials website (www.clinicaltrials.gov) indicates another five currently registered trials investigating ASC in cardiac disease states [94]. Interested industry representatives are also becoming involved with such research. Cytori Therapeutics, Inc. is currently sponsoring three European clinical trials to evaluate the use of ASC in acute MI and chronic myocardial ischaemia [95]. These include two safety and feasibility trials (APOLLO and PRECISE trials)

as well as one randomised, placebo-controlled, double-blind trial enrolling up to 360 people from 35 centres for treatment of ST-elevation acute MI via intracoronary delivery of ASC (ADVANCE trial).

Overall, there is a growing body of evidence in laboratory and animal models that ASC can improve cardiac function via cell differentiation and through paracrine pathways [96, 81] and we anticipate an explosion of human trials in the coming years. The results of the trials which are currently recruiting will not be expected for several years. Potentially, they will show that ASC could provide an exciting new treatment pathway for cardiac disease. However, the differentiation pathway of ASC remains poorly detailed, so many of the results remain controversial [81]. Also, treatment of acute MI with autologous ASC is also hindered by the fact that optimal treatment may require such a number of cells that weeks of in vitro culture may be required, delaying the onset of therapy [82]. Ongoing research, including assessment of the suitability of allogeneic ASC in acute situations, will contribute to clinical translation of ASC research in this field.

Neuronal Regeneration

There is some evidence that ASC might be able to differentiate along a neuronal lineage. Obviously that would be valuable clinically, as neurons have a limited capacity for regeneration or repair, and endogenous neural stem cells (in the central nervous system (CNS)) and Schwann cells (SC) (in the peripheral nervous system (PNS)) have limited availability and therefore limited applicability [10]. While other sources of stem cells for neuronal repair are clearly necessary, the use of mesenchymal origin adult stem cells of any kind would be unlikely to be a useful source, as such transdifferentiation is somewhat unlikely to occur without dedifferentiation of the cells first occurring.

However, there is a growing body of evidence supporting neuronal differentiation of ASC to a limited degree. Neuronal differentiation was first attempted from BMSC, and protocols for ASC transdifferentiation have been largely adapted from BMSC protocols for the same [97]. Phenotypically similar cells have been produced from ASC in vitro with morphologic similarities and evidence of neuronal gene expression [11, 98]. In a murine model of sciatic nerve injury, ASC cultured under neuronal differentiation conditions for 2 weeks were seeded into a fibrin nerve conduit to encourage sciatic nerve regeneration. An improvement in regeneration distance compared with empty conduits was shown with both BMSC- and ASC-seeded conduits, which shows that they support axonal regrowth, but regrowth occurred in the empty conduits also [99]. Similarly, Erba and colleagues showed axonal outgrowth in a rat model of sciatic nerve injury with ASC in a nerve conduit. However, few viable ASC were still present 14 days after transplantation, which suggests that the results may be related to endocrine or paracrine effects of the ASC rather than transdifferentiation of the cells themselves [100]. Overall, the use of ASC in sciatic nerve injury shows some evidence of improvement [101] but no clear evidence of neuronal differentiation of the ASC themselves.

A review of published trials looking at the use of MSC for neurological disorders showed 25 previously published, none of which utilised ASC as the MSC of choice, and 54 active, recruiting or completed but unpublished trials, of which only one specifies adipose as the MSC of choice for the trial [102]. Embryonic stem cells (ESC) and adult neural stem cells have been shown to produce functioning neurons [103], but no study has shown this from ASC so far [97]. It seems that the clinical use of ASC for the treatment of central and peripheral nervous system is still a long way off and that research into the effects of ASC on nerve regeneration will need to address the question of true transdifferentiation.

Treatment of Immunological Disorders

MSC are known to cause immunomodulation by inhibiting different immune cell types. They act on T-cell lymphocytes to inhibit proliferative responses, reduce production of inflammatory cytokines and induce anergy (tolerance); they inhibit the cytotoxicity of natural killer (NK) cells as well as inhibiting cytokine secretion, and they downregulate co-stimulatory molecules on dendritic cells, among other actions [16, 104, 105]. The mechanism of these actions is not yet fully understood [104]. Consequently, ASC, despite expressing HLA class I and having inducible HLA class II antigens, are poorly immunogenic and could potentially be transplanted between non-identical individuals [106]. Consequently, harnessing these properties to modulate the onset or progression of chronic immune-mediated diseases has become an area of great research interest with regard to ASC. This includes a wide variety of disorders, such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA), graft-versus-host disease (GVHD), autoimmune thrombocytopenic purpura (AITP), among others.

In vitro studies, commonly using mouse models, have underpinned the recently published phase I trials and clinical case reports. A mouse model of GVHD showed that intravenous (IV) infusion of ex vivo expanded ASC was able to control otherwise lethal GVHD, although the infusion has to occur at or close to the time of the haematopoietic stem cell grafts and potentially required multiple injections to be effective [107]. Similarly, serial IV injection of ASC every fortnight resulted in the reversal of immunologic dysfunction in

mice with SLE [15]. No side effects were found in this study, although it was noted that it was preferable to begin the injections before the onset of symptoms of the disease, which is not necessarily possible in the clinical situation. ASC have been shown to slow the progression of Huntington's disease in a mouse model, as shown by behavioural and striatal degeneration [108]. Both human and murine MSC have been shown to improve MS outcomes in murine models, presumably via the inhibition of the immune-mediated components of tissue destruction [109]. Another disease with potential clinical improvement from ASC is colitis. In a mouse model of induced colitis, intraperitoneal infusion of human ASC increased survival and significantly improved the severity of the colitis [110]. Subsequent research by the same group showed that the therapeutic effect was mediated by down-regulation of both T-lymphocyte-driven autoimmune and inflammatory responses, resulting in a decrease in a wide range of pro-inflammatory cytokines and chemokines [111].

Several groups have published early human clinical data in this field, most commonly using ASC on compassionate grounds in patients who had exhausted their other, more traditional, treatment options [14, 112, 113]. The results of the phase I clinical translational trials have so far been promising. Ra's group performed a pre-trial safety assessment of IV infusion of human ASC into immunocompromised mice and found that, even at the highest doses of injection, there were no side effects [114]. They then went on to perform a phase 1 trial of IV infusions of ASC for a variety of different autoimmune diseases (including MS, RA, atopic dermatitis, autoimmune hearing loss and polymyositis) in ten patients [14]. The patients ranged in age from 19 to 67 years. Follow-up ranged from 2 to 13 months, during which time no adverse events were noted and all patients were assessed clinically and found to show improvements in their symptoms. Another group used intrathecal and IV infusions of unpurified SVF, rather than ASC, to treat MS in three patients [115]. Unfortunately, the use of the SVF means that the role of the ASC in the clinical effects cannot be ascertained with certainty. However, they felt that the results were promising and warranted larger clinical trials. A single case report of a 21-year-old patient with chronic AITP, previously treated with corticosteroids, immunosuppression and a peripheral blood stem cell transplant, was subsequently treated successfully with ASC from his brother. Follow-up at 39 months showed sustained resolution of his disease. The same group has also reported a phase I trial of six patients with steroid-refractory acute GVHD, whom they treated with IV ASC infusions [112]. Two patients received ASC from matched family donors, while the others received ASC from unrelated mismatched donors. The acute GVHD disappeared completely in five of the six patients. The remaining patient showed no obvious response to the treatment and died of multiorgan failure. One of the five responders subsequently died from relapse of leukaemia. However, since this form of disease is associated with a high morbidity and mortality [116], these results were felt to be good, and the use of ASC is considered to be a promising potential therapy. These latter two studies from Fang and colleagues are also interesting as some of the first to be published for utilisation of allogeneic, rather than autologous, ASC.

Preliminary clinical data appears to support the cautious use of ASC in a variety of human chronic immune-mediated disorders. At least three multicentre, phase I/II clinical trials are currently recruiting participants to evaluate the safety and feasibility of ASC in the treatment of MS and GVHD. Further trials are likely if the reported outcomes continue to be positive.

Safety Issues with Adipose-Derived Stem Cells

The use of autologous fat for a number of different applications has gained in popularity over the past decade. FIs and ASC have shown themselves to be versatile workhorses for soft tissue reconstructions and potential life changers in organ regeneration. However, despite the many publications on the uses of ASC, no long-term safety data has yet been seen, given the relatively short term over which ASC have been isolated and used clinically, which is only about a decade at this stage. Clearly autologous fat and ASCs are biologically active substances, exerting not just volume effects but also paracrine and endocrine effects due to the secretion of metabolically active hormones, cytokines and growth factors [117]. These characteristics have fuelled concerns about the potential of injected ASC to stimulate the growth of cancer. MSC are thought to contribute to tumourogenesis by both direct and indirect mechanisms [118, 119]. Direct effects include contributing to the formation of tumour stroma and promoting epithelial to mesenchymal transition. Indirect effects include enhancing angiogenesis and reducing the natural immune response, both of which may provide a more favourable environment for cancer growth. ASC are also immunosuppressive. Despite expressing human leukocyte antigen (HLA)-1 and having inducible HLA-2, they are poorly immunogenic and can engraft across immunologic barriers [120]. To this end, ASC have been shown in animal models to home to areas of chronic inflammation and assist in the transformation of gastritis to gastric cancer [118]. Similarly, BMSC co-injected into immunocompetent mice with a weak melanoma cell line favours the development of melanoma over injection of the melanoma alone [121], while co-injection of MSC with breast cancer cell lines seems to promote tumour metastasis [122]. Other laboratory studies are more supportive, with injection of labelled ASC subcutaneously into a murine model showing not only no signs of de

novo cancer development at 1 year but also no evidence of any of the injected ASC persisting at that time point [123].

The most important question is how relevant are these in vitro results to our clinical practice? It seems unlikely that murine results are directly translatable into human outcomes. Similarly, co-injection of tumour cells with ASC would never occur in clinical practice. However, these early results offer a word of caution, and it is essential that we confirm the safety of our practices to protect our patients. To add to the concern, a single case report was published in 2010 showing recurrence of an osteosarcoma following three episodes of FI to the site [124]. The patient had osteosarcoma at age 17 years and was treated with wide local excision and radiotherapy. After 13 years of being tumour free, she underwent the FI procedures to repair the defect. She then developed recurrence of her cancer 18 months later. While it is impossible to directly attribute the recurrence to the FIs, the authors note that only 5 % of patients with disease recurrence have local recurrence, and the median time to recurrence is 6–24 months, making such late recurrence highly unexpected. The greatest volume of human data currently available regarding the injection of fat or ASC into a site of cancer or a cancer-prone region is in the field of breast reconstruction. However, the data are generally providing low-level evidence only, such as retrospective observational studies with or without controls. Reitjens and colleagues retrospectively reviewed their breast cancer patients who had undergone FIs [31]. Of the 158 patients who underwent 194 FI procedures, they reported only 7 immediate complications (3.6 %) and one local relapse. However, their minimum follow-up was only 6 months, and the average length of patient follow-up was not stated, meaning that no useful long-term safety data can be drawn from this paper. Rigotti's team from Italy have also published their outcome data to help determine the oncological risk of FI following mastectomy [125]. They retrospectively reviewed 137 patients who had between two and four FI procedures. Their follow-up was longer with a median of 56 months. There was no control group; however, they were retrospectively matched with patients who did not undergo lipofilling. Conclusions were drawn by making comparisons between the locoregional recurrence rate in the period from mastectomy to FI (period 1, average 23 months) and FI to completion of follow-up (period 2). The authors found that the relapse rate was higher in the first period and concluded that FI did not increase the relapse rate and seemed to be safe. However, this methodology is flawed by the fact that the majority of relapses occur in the first 2 years after surgery, independent of any FI [126]. Also, patients undergoing breast-conserving therapy (BCT) were not included. This may be the group who are at highest risk of recurrence from this treatment. The reason for this is twofold. Firstly, the most likely site for recurrence in BCT is the quadrant of the primary tumour [127], which would be the site of the defect for treatment with ASC or FI. Secondly, recent evidence shows that the residual breast tissue on the side of the primary breast cancer may have tumour-initiating mutations [117]. Moinfar showed that tumour suppressor gene loss of heterozygosity mutations was seen at multiple loci in the breast stroma [128], which may make this tissue more susceptible to tumourogenesis.

Overall, there is no good evidence at present showing that ASC cause or promote cancer in clinical use in humans. However, the supporting evidence is of poor quality. Only well-designed, randomised-controlled trials with long-term follow-up will properly address this issue.

Future Directions of Adipose-Derived Stem Cell Therapies

While most non-medical people regard adipose tissue as unwanted and undesirable, it has recently become very popular in medicine as a prolific source of multipotent adult stem cells. Given the increasing abundance of fat in the population, combined with the relative abundance of ASC, their ease of harvest and culture, it is likely that the use of ASC in clinical practice will only increase from here [23]. Fat grafting procedures have already gained popularity over the past decade, and it is likely they will continue to do so. While early reports show positive clinical outcomes, it will only be with well-designed, randomised-controlled studies and long-term follow-up that we can ensure the safety of these procedures for our patients. In the interim, it is the author's opinion that such procedures should be undertaken with caution, with appropriate informed consent and with the intention of long-term patient surveillance to detect and document any potential adverse events.

Regarding transdifferentiation of ASC, the real question which still needs to be definitely answered is whether transdifferentiation or dedifferentiation of ASC actually occurs. If not, then the use of ASC in nerve regeneration or repair seems unlikely to be useful. Overall, it seems that levels of ASC undergoing transdifferentiation are low compared with mesenchymal differentiation pathways, with inefficient protocols currently in use and poorly or non-functioning cells being produced thus far. Current evidence seems to suggest that cell fusion (i.e., fusion of the ASC with the host or native cells) may explain the results of some of the current research findings [129, 130]. It seems unlikely that the differentiation of ASC into neuronal cells using these methods will be clinically efficacious in the near future. However, given the paucity of neural stem cells, work in this area is likely to continue. The future of transdifferentiation of ASC may lie in direct differentiation using transcription factors. Mouse fibroblasts have been directly converted into neuronal cells by expression of three factors, a technique that could be

applied to ASC in the same way that ASC have been used to make induced pluripotent stem cells rather than fibroblasts [131, 132]. Emerging technologies for transiently expressing factors required for transdifferentiation will make this a clinical reality in the near future [133].

Another future direction for ASC research is in the field of gene therapy. Potential uses being investigated include harnessing their homing and engrafting ability to deliver growth factors or cytokines to chronic wounds [134, 135], delivery of prodrugs to cancer sites [136] or addition of pancreatic genes to treat diabetes by promoting insulin expression [137]. Clearly, ASC offer exciting potential solutions for many challenging human diseases, including cardiac disease, organ regeneration and refractive immunological disorders. The keys to their future clinical use include detailed analysis of the control pathways of differentiation and the development of efficient, reliable differentiation protocols. Long-term, large-scale human trials will be necessary to confirm the safety of their use, as well as their long-term efficacy. Rapid progress is expected in this field over the coming years.

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14 Potentialities of Adipose-Derived Mesenchymal Stem Cells Collected from Liposuction for Use in Cellular Therapy

Patricia H.L. Pranke and Pedro C. Chagastelles

Introduction

The increased use of stem cells in the last decade has taken place for two main reasons. Firstly, it was discovered that more sources of stem cells exist besides those from the bone marrow and, secondly, that these cells have been studied for the treatment of other diseases and not only for hematopoietic disease, through the use of bone marrow transplants.

Although bone marrow transplants have produced a successful source of stem cells for almost half a century, one of the main limitations in their use is the very invasive method for collecting stem cells from the bone marrow or the need to submit the donor to apheresis, which is also an uncomfortable procedure. It has limited the availability of stem cells for use in many other diseases and patients. The new discoveries concerning stem cells have broken paradigms, and the studies around this topic have increased considerably since the end of the last century. The discovery that stem cells can be used for many different diseases, such as diabetes; heart, liver, and neuronal diseases; and others, as well as the fact that the human body has stem cells in basically all tissue, such as the teeth, umbilical cord, adipose tissue, and others, has made this science one of the most fascinating and promising areas of study of the new millennium.

Adipose Tissue

The accumulation of fat in obese or overweight people at the subcutaneous and visceral sites can cause several health issues and an increased risk of mortality. In some cases the loss of weight cannot be achieved only by diet, exercises, or medications and more drastic procedures need to be taken. In these cases, bariatric surgery is the better alternative to achieve long-lasting weight loss and successfully decrease the risk of associated illness, such as diabetes and cardiovascular diseases [1]. A few years after bariatric surgery, patients experience an expressive weight loss and many of them feel the need of aesthetic plastic surgery to get rid of the excess skin and fat that remains. In some cases it is not only for aesthetic reasons but also to improve life quality in terms of loss of skin elasticity, posture and equilibrium problems, and dermatitis due to the excess of skin [2, 3].

The major function of adipose tissue is the storage of energy in the form of lipids but it also functions as a thermal isolator and an endocrine organ, secreting several hormones. Adipose tissue is mainly composed of adipocytes, but other cell types, such as blood cells, endothelial cells, and fibroblasts, are also found [4, 5]. Fibroblasts and mesenchymal stem cells are closely related cell types to the point that some groups suggest that they are the same cell [6, 7]. The adipose tissue is highly vascularized and the endothelial cell network increases as fat accumulates [4].

Stem Cells from Adipose Tissue

Mesenchymal stem cells (MSCs) or mesenchymal stromal cells are found in many tissues and organs throughout the organism and are located in the perivascular niche [8, 9]. These cells have specific in vitro characteristics, such as the ability of attaching to plastic surfaces, the expression of a known set of surface proteins, and the ability to differentiate into adipocytes, osteoblasts, and chondroblasts [10]. Adipose-derived stem cells (ADSCs) are the mesenchymal-like counterpart found in adipose tissue (Fig. 14.1). They share many of the characteristics of other MSCs from the bone marrow, teeth, umbilical cord, and placenta, for example. ADSCs differentiate in vitro in endothelial cells, proved by the expression of CD31 and von Willebrand factor, and can form new vessels in vivo. It is postulated that MSCs are

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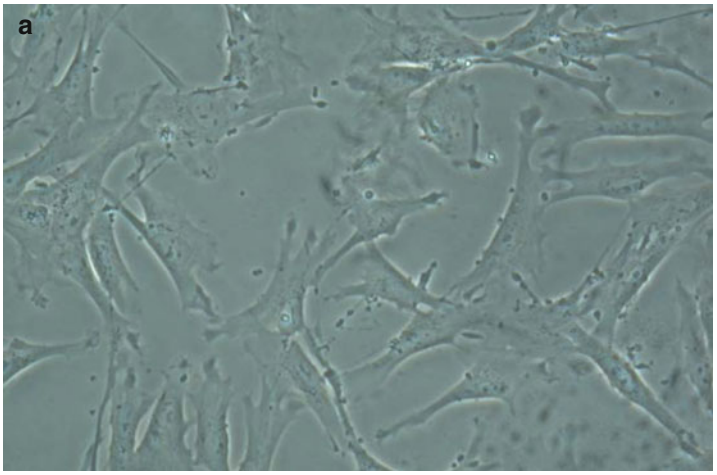
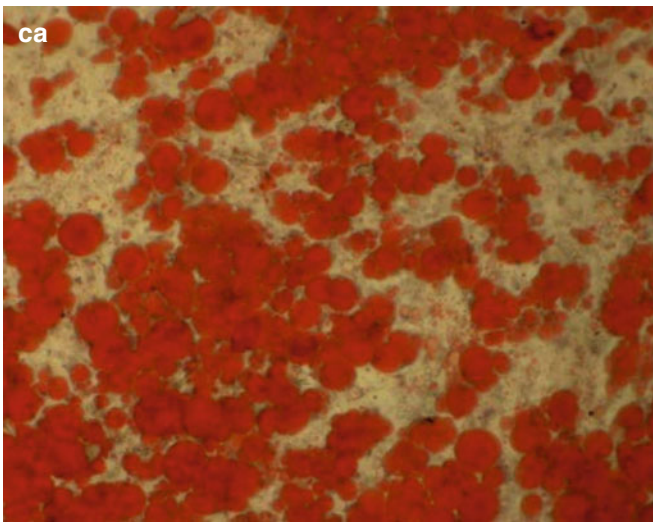


b	Surface marker
Positive	CD73, CD90, CD105
Negative	CD14 or CD11b, CD34, CD45, CD19 or CD79alpha and HLA–DR



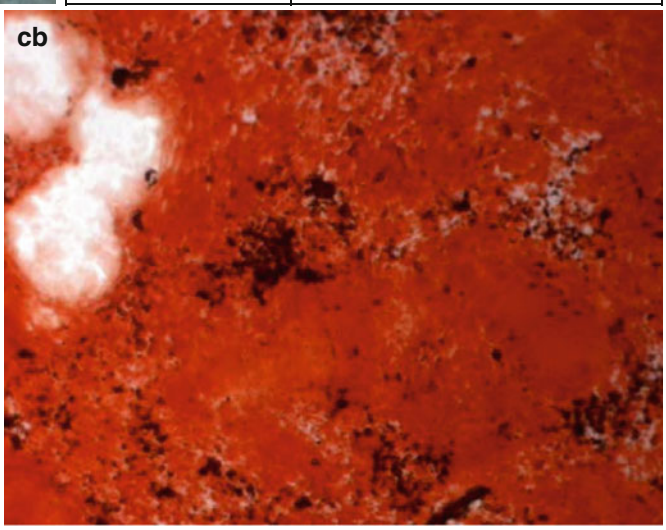


Fig. 14.1 (**a**) Morphology (**b**) panel of cell surface markers and (**c**) adipogenic (**ca**) and osteogenic (**cb**) differentiation of adipose-derived mesenchymal stem cells

involved in vessel stability and tissue homeostasis by many mechanisms and pathways [11].

ADSCs have the advantage over MSCs from other sources, because of the high yields that they can render. Fat tissue has 500 times more stem cells per gram of tissue compared to the bone marrow [12] and much more tissue available than the tooth pulp or umbilical cord. This allows a shorter time for in vitro expansion to achieve the necessary cell numbers for clinical applications, decreasing the period in culture and allowing for cell therapy for acute cases.

Stromal Vascular Fraction (SVF) X Cultured ADSCs

The isolation of stem cells from adipose tissue can be performed by a procedure that evolves collagenase digestion. For the isolation, it is possible to use liposuction material or resected fat tissue cut in small pieces [13]. After the isolation process two therapeutic approaches can be chosen: (i) the use of freshly isolated cells, also called stromal vascular fraction (SVF), which are composed of an enriched fraction of adipose-derived stem cells but still containing other cell types, such as endothelial cells and blood cells, and (ii) the use of adipose-derived mesenchymal stem cells after in vitro culture. However, there is so far no consensus about which approach would bring the best results.

The advantages of using cultured ADSCs are the possibility of transplanting pure stem cell populations and, thanks to the high in vitro proliferative capacity, the expansion of large numbers of cells for therapy. On the other hand, the period of in vitro cell expansion could be potentially deleterious to the cells [14]. It is known that long periods of culture can induce senescence [15, 16], increase oxidative stress, and raise the risk of genetic alterations in the cells, which include punctual mutations and chromosomal anomalies. For this reason, the majority of clinical protocols only expand cells for a few passages before transplanting them to patients. Two studies described the appearance of transformed cells from adipose-derived stem cells after long periods of culture, but further

investigations proved these to be cross contaminated with epithelial cell lines [17, 18]. Works reported aneuploidies in cultured MSCs [19, 20] but the cells did not develop into a tumor after implantation in immunodeficient mice [21]. In addition, there is no evidence until the present day of tumor formation of transplanted MSCs from any source after clinical application.

The isolation and culture of ADSCs for clinical application requires a series of rules, and all procedures need to be carried out in a special environment by trained staff to guarantee safety and minimize the risk of viruses or other hazardous contaminants to the culture. Production of cells for therapy must mainly follow the same regulations as for drug production [22] although the regulations vary from country to country. The isolation and expansion process of ADSCs uses several animal-derived components, such as collagenase, trypsin, and serum that could transmit diseases to humans. All these components must be screened and certified for clinical application, increasing significantly the cost of these products. In addition, attempts have been made to create culture conditions that allow the expansion of mesenchymal stem cells in the absence of animal components, called xeno-free culture. There are alternatives that replace even more efficiently the classical and widely used fetal calf serum, such as human serum or platelet-rich plasma [23]. The expansion of high numbers of cells is being scaled up in automated bioreactors that control parameters, such as temperature, pH, and glucose, and which minimize the operational risks.

Clinical Application of ADSCs

The potentialities of ADSCs in medicine are the same for MSCs from the bone marrow since they share many of their basic characteristics. Preclinical application of MSCs and ADSCs has been studied for more than 10 years and the vast majority of the studies show improvements for a wide range of diseases. Some of the mechanisms responsible for the improvements are already known, and the principal one seems to be by paracrine secretion of soluble factors, which acts by improving angiogenesis, inhibiting apoptosis, and controlling inflammation in target tissues [24]. Since the first description of ADSCs by [25], the number of research groups and publications that use these cells has increased enormously. The translation from basic and preclinical studies to clinical trials has occurred really quickly for ADSCs since they were initially described. Research at the ClinicalTrial.gov website found a total of 41 studies that employ ADSCs or stromal vascular fraction in cell therapy. The majority of the trials use autologous cells (90.2 %) and only 9.8 % employ allogeneic cells. Thirty-eight studies are phase I/II, four are phase III, and only one is a phase IV trial. Table 14.1 describes the clinical trials, the results of which have been published. The most common diseases treated are fistulas (Crohn's disease) and others such as dermatitis, facial tissue defects, urinary incontinence, and autoimmune diseases, among others. In all cases the safety of the therapy was attested.

Table 14.1 List of clinical trials that use stromal vascular fraction or adipose-derived stem cells

Disease	Phase	Cell type	Major findings	Reference
Crohn's fistula	I	ADSCs	Safe procedure	[26]
Crohn's fistula	I	ADSCs	Safe procedure	[27]
Radiodermatitis	I	Vascular stromal fraction	Improvement or remission of the lesions	[28]
Perianal fistula	I	ADSCs	Safe procedure	[29]
Facial lipoatrophy	I	ADSCs + fat tissue	Safe procedure	[30]
Breast augmentation	I	ADSCs + fat tissue	Cyst formation and microcalcification in some patients	[30]
Crohn's fistula	I	Stromal vascular fraction or ADSCs	Safe procedure	[31]
Perianal fistula	II	ADSCs	Safe and effective treatment. Increased rate of fistula healing in cell-treated group	[32]
Urinary incontinence	I	Stromal vascular fraction	Safe procedure. Apparent improvements in several parameters	[33]
Perianal fistulas	II	ADSCs	Safe treatment. Low proportion of cell-treated patients remained free of the recurrence of fistula	[34]
Idiopathic pulmonary fibrosis	I	Stromal vascular fraction	Safe procedure	[35]
Autoimmune hearing loss, multiple sclerosis, polymyositis, atopic dermatitis, and rheumatoid arthritis	I	ADSCs	Safe procedure	[36]
Facial tissue defects	I	Stromal vascular fraction	Safe procedure	[38]
Spinal cord injury	I	ADSCs	Safe procedure	[37]

ADSC for Heart Disease

Adipose-derived stem cells can engraft and turn into cardiomyocytes [39] and endothelial cells [40] in vivo, two cell types to be replaced in injured hearts. However, the frequency of differentiated cells does not seem to explain the overall improvements in animal models. The most acceptable explanation is the paracrine effects of MSCs or, in other words, the trophic molecules secreted by the cells, which play a central role in myocardial recovery [41]. The first clinical trials using bone marrow mononuclear cells focused on cardiac diseases. Despite the good results in initial studies with a small number of patients, studies that tested the efficacy of these cells found little or no improvement in functional parameters for different cardiac diseases [42, 43]. Clinical trials are in the process of testing the safety and efficiency of cultured MSCs. The MyStromalCell Trial is currently recruiting participants of phase II, randomized, double-blind study, which will evaluate the safety and efficacy of ADSC in patients with chronic ischemic heart disease.

ADSCs for Autoimmune Diseases and GvHD

One of the properties by which MSCs and ADSCs play their roles is through the modulation of the immune system. With this in mind, the ADSCs have the potential for the treatment of autoimmune disease [44]. MSCs may respond according to the environment and could act as pro- or antiinflammatory cells, depending on the stimulus from the environment. They can also have antigen-presenting proprieties [45]. MSCs have been studied with interesting results in animal models of type I diabetes [46], autoimmune encephalomyelitis [47], and rheumatoid arthritis [48], among others. MSCs are also effective for the treatment of graft versus host disease (GvHD) in humans [49]. ADSCs have comparable immunosuppressive proprieties presented by MSCs from the bone marrow [50] and a presumed potential to treat these diseases. At the moment, there is one phase I/II clinical trial recruiting patients for the treatment of GvHD with allogeneic ADSCs registered at ClinicalTrials.gov, and no study has been published so far.

ADSCs for Neurologic Diseases

Cell therapy for the nervous system is so far the most challenging and expectative field. The reason for this is the complexity of this system and the diversity in the diseases types that have no available treatment. The approaches for the treatment will vary, for example, according to the illness and time of disease or injury. In the case of stroke or brain lesion, it has been reported that the therapy is more effective in acute cases, when cells are administered a few days after the injury and are less likely to have an effect in chronic patients [51]. It is perfectly explained by the paracrine effects of MSCs that preserve and protect cells from damage through the secretion of antiapoptotic and antiinflammatory molecules. For spinal cord injury, for example, another reason why MSCs have better effect in acute and not chronic patients is due to the fact that after the glial scar has been formed, it is more difficult for the stem cells to perform. The glial scar acts as a physical barrier and as an inhibiting environment for axonal regeneration and remyelination [52, 53].

In the case of Parkinson's disease, on the other hand, the goal of the therapy is to replace lost dopaminergic neurons. The ability of ADSCs in turning into neuronal cells is still controversial. Despite the fact that some studies show that differentiated cells present morphological and phenotypical characteristics of neuronal cells, such as neurons, astrocytes, and oligodendrocytes, they do not have all the functionality expected [54]. ADSCs have proved to be a safe procedure in trials for spinal cord injury. Additional studies are recruiting patients to test these cells in phase I/II clinical studies for other diseases, such as Parkinson's disease, multiple sclerosis, brain lesions and stroke, (ClinicalTrials.gov).

ADSCs and Tissue Engineering

Apart from use in cell therapy, stem cells have also been explored in the promising potential field of regenerative medicine. This new research approach combines elements from other areas to create tridimensional organs and tissues by the combination of scaffolds, cells, and trophic factors. Material science permits the creation of biomaterials with a range of physicochemical parameters, which confers specific mechanical and structural proprieties to the scaffolds.

It is especially important in cases where large quantities of tissue are lost and the goal in the future is to replace whole nonfunctional organs. As mentioned before, ADSCs can easily differentiate into cells from a mesodermal origin; consequently they can be used to originate bone [55], cartilage [56], and fat [57] substitutes. Application of ADSCs in tissue engineering, or regenerative medicine, is still taking its first steps and the clinical studies are limited to case reports [58]. Examples of this are results described by [59] who used adipose-derived stem cells and beta-tricalcium phosphate granules to create cellularized scaffolds for the treatment of large calvarial defects or by Mesimaki and colleagues [58] who also used beta-tricalcium phosphate granules, ADSCs, and BMP-2 in a microvascular plat to create a functional bone graft. One of the big challenges of tissue engineering is the creation of vascularized networks throughout the scaffold, which permit nutrient and oxygen diffusion and a quicker blood supply to avoid massive loss of transplanted cells in the posttransplanting period. Taylor [60] published

the results of a case report which constructed engineered bone by the combination of bone allograft, adipose-derived stem cells, bone morphogenetic protein-2, and periosteal graft. The results presented showed long-term bone stability and no significant chirurgical side effects, despite an expressive postoperative swelling.

Conclusion

Adipose tissue is a rich source of mesenchymal-like stem cells for application in cell therapy and tissue engineering. It is relatively easy to be isolated and renders a high yield compared to other sources, which facilitates the application of these cells for therapeutical purposes. Data from clinical applications of ADSCs and SVF to date now permit us to conclude that the administration of these cells either intravenously or locally is a safe and feasible procedure. The majority of the clinical trials are in phase I and II and do not permit the formation of conclusions about the efficiency of the transplanted cells. The real potential of using fat-derived mesenchymal stem cells for therapy is still uncertain, and it will only be ascertained with multicentric, randomized, double-blind studies to be performed in the near future.

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Part IV

Other Important and Emerging Sources of Stem Cells

Stem Cells from Dental Tissue for Regenerative Dentistry and Medicine

15

Patricia H.L. Pranke, Luciano Casagrande, and Simone Bonato Luisi

Introduction

Tissue engineering is a multidisciplinary science that aims to develop biological substitutes for tissues and organs in order to restore their functions in cases of injuries and deformities. In recent years, dentistry has explored the potential of tissue engineering through basic and translational research in order to be able to repair oral tissue damaged by biological substitutes. This new science is based on the balanced use of responsive cells, capable of differentiating in tissue of interest; biocompatible matrices, those that will support cell growth and mimic the extracellular matrix; and bioactive molecules responsible for morphogenetic signals. Currently, stem cell research has grown significantly, due to the recognition that therapies based on stem cells have the potential for treating patients under different conditions, such as bone tissue deficiency and ischemic heart disease, and even in severe conditions, such as Alzheimer's disease and leukemia. Current evidence has demonstrated that stem cells are found in certain niches and some tissues contain more stem cells than others. Since the discovery that dental tissue can become a source of mesenchymal stem cells, a vast field of research has opened and there are promising opportunities for regenerative therapies. Due to its favorable characteristics, such as low risk of tumor formation, sources of relatively easy access, and lower ethical issues related to their use, stem cells from dental tissue are being considered as promising for clinical use in both medical and dental applications. The areas, such as endodontics, periodontics, oral surgery, and dental implants, are increasingly exploring the potential of dental tissue engineering in search of better and more advanced treatment options for their patients. Thus, this chapter aims to introduce the basic concepts of tissue bioengineering in dentistry, focusing on the isolation methods and characteristics presented by stem cells from dental tissue as well as its potential use in cell-based therapy.

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Sources of Mesenchymal Stem Cells in Dental Tissue

Stem cells are commonly defined as clonogenic nonspecialized cells that have the ability to divide continuously by means of self-renewal and generate progenitor cells that differentiate into several cell lines [1]. These characteristics make a distinction between stem cells and restricted progenitors (e.g., circulating endothelial cell progenitor cells) or differentiated cells, which have a more narrow developmental potential and a reduced ability to proliferate [2]. These cells can be classified according to their origin, such as embryonic, adult, and, most recently, induced pluripotent stem cells (iPSCs). The embryonic stem cells (ESCs) are found in the early stages of embryonic development [3]. The cells in the embryos until 3 days are considered totipotent, and the cells from the inner mass of the blastocyst are pluripotent cells. Despite the great regenerative potential of these cells, their isolation and use face ethical and legal barriers [4] which reduce their attractiveness within the regenerative therapies [5].

The ethical issues surrounding the use of embryos to obtain stem cells and the possibility of these cells undergoing transplant rejection in patients generated the idea of

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producing pluripotent cells from non-stem cells of the patient himself/herself, reducing the risk of transplant rejection. It has been demonstrated that somatic cells can be reprogrammed by transferring their nuclear contents into oocytes [6], or by fusion with ESCs [7, 8]. This possibility indicates that eggs and ESCs contain factors that confer totipotency or pluripotency. It was then assumed that these factors would also have a crucial role in the induction of pluripotency in somatic cells. This hypothesis was confirmed by using fibroblasts from mice, transfected with transcription factors responsible for the maintenance of the pluripotency to generate iPSCs [9].

Adult stem cells (ASCs) are stem cells found in several different formed tissues, including the bone marrow, blood, brain, cord blood, and many other organs, which give rise to different tissues. There are different types of ASCs with different properties. One of the most studied types of ASCs is the mesenchymal stem cells (MSCs). These cells are considered to be an attractive source of cells for regenerative therapies [10, 11] because they have unique plasticity when exposed to different environments. Furthermore, clonogenic cells are capable of self-renewal and differentiation into multiple lines [12]. The first MSCs to be isolated and characterized were from the bone marrow (BMMSCs) [13]. These cells showed that they have the potential to differentiate into osteoblasts, chondrocytes, adipocytes, and myelosupportive fibrous stroma [10]. MSCs were isolated from various tissues, such as the brain, skin, hair follicle, skeletal muscle, and pancreas [14–16].

In 2000, MSCs were isolated from the pulp tissue of permanent teeth [17]. This discovery opened up a wide range of possibilities for the application of regenerative therapies based on the use of stem cells in oral tissue engineering. So far, five types of human stem cells of dental origin have been isolated and characterized: stem cells from the dental pulp (dental pulp stem cells – DPSCs), stem cells from the exfoliated deciduous teeth (stem cells from human exfoliated deciduous teeth – SHED), stem cells of the periodontal ligament (periodontal ligament stem cells – PDLSCs), stem cells from the apical papilla (stem cells from apical papilla – SCAP), and progenitor cells of the dental follicle (dental follicle progenitor cells – DFPCs). These populations of dental stem cells share characteristics common to other populations of mesenchymal stem cells [10].

The plasticity of stem cells is assessed by their capacity for self-renewal and proliferation and differentiation in many different directions. The comparison of the potential of proliferation, differentiation, and osteoinductive capacity of BMSCs, DPSCs, and SHED showed a superior cell proliferation in favor of the pulp and in particular of the primary teeth [18]. The great proliferative capacity and differentiation of stem cells from pulp tissue of primary teeth have raised interest from many areas. Recent evidence suggests the possible usefulness of dental pulp stem cells in the treatment of diseases and disorders such as muscular dystrophy, bone defects, and large jaw calvaria. It also aids in the repair of lesions of the cornea and shares similar characteristics of the liver cells. The dental pulp stem cells' plasticity will be commented on afterwards [19, 20].

The Role of a Microenvironment for Pulp Stem Cell Differentiation: Growth Factors and Cytokines Determining the Stem Cells' Fate

Growth factors and bioactive molecules are morphogenic proteins or factors which bind to specific cell membrane receptors and trigger a variety of signaling pathways that coordinate in an orderly manner all cellular functions. Several growth factors are used to control the activity of the stem cells by increasing the rate of proliferation, inducing differentiation into another cell types, or stimulating the cells to synthesize and secrete a mineralized matrix. Most of these factors operate at very low concentrations although they are sufficient to induce changes in gene expression [21].

These molecules play a critical role during embryonic development by determining the fate of the stem cells and regulating the development of all organs and tissues. The same factors that modulate the growth of embryogenic tissue can also be used therapeutically to guide the differentiation of stem cells to various destinations and coordinate the cellular processes that ultimately result in the generation of a new organ or tissue. More specifically, there are many similarities between the factors involved in tooth formation and the molecules that regulate the formation of reactionary dentine [22].

The dentine matrix contains growth factors and cytokines (members of TGF-b superfamily, BMP, FGF, IGF, VEGF, Hedgehog, Wnt, and other genes and transcription factors) that are sequestered during dentinogenesis [23, 24]. Following physiological stimulation or injury, such as caries, trauma, and operative procedures, these molecules are released by acids with other extracellular matrix components [25]. Interestingly, when these molecules are released from the dentine, they are fully capable of inducing cellular responses, such as those which lead to the generation of reactionary dentine [26–29]. The arrangement of the tubular dentine facilitates the movement of growth factors released by etching agents, carious lesions, or pulp-capping materials from the dentine matrix to the pulp.

Even after its complete formation, the dentine-pulp complex retains the ability to respond to different stimuli [29–32]. Dentinal repair occurs through the activity of specialized cells, called odontoblasts, which are thought to be maintained by an undefined precursor population associated with pulp tissue [29].

Under injury of medium intensity, such as superficial dentin carious lesion, trauma, and restorative procedures, the odontoblasts increase their secretory activity, producing mineralized matrix. However, this activity is directly related to the maintenance of a viable population of cells near the injury site,

resulting in the deposition of mineral matrix and the growing distance between the carious lesion and pulp tissue [33].

With the progression of carious lesions, the layer of odontoblasts underlying carious lesions succumbs, and with prevailing favorable conditions of pulp vitality, a new cell population is recruited to the region and begins to produce mineralized tissue [32]. These events involve the recruitment of dental pulp stem cells (DPSCs) and their differentiation into functional odontoblasts, which secrete in mineralized matrix [26, 34].

Studies on mechanisms underlying the differentiation of dental pulp stem cells are critical for the understanding of the biology of dental tissue engineering. Recent research has produced important evidence concerning the role of bioactive molecules that are present in the dentine and which induce differentiation of pulp stem cells into odontoblasts [35]. It was observed that when SHED were seeded onto a polymeric-based scaffold made inside the pulp chamber of a dentine disk treated with EDTA, the cells expressed odontoblast differentiation markers, such as DMP-1, DSPP, and MEPE. In contrast, when SHED were seeded onto scaffolds surrounded by a deproteinized dentine disk (long-term treatment of sodium hypochlorite), no evidence of differentiation was found. This finding also demonstrated that that dentine-derived BMP-2 is required to induce the differentiation of SHED into odontoblasts.

It has been shown that stem cells from human exfoliated deciduous teeth can also differentiate into functional odontoblasts and endothelium [36]. When SHED were seeded onto tooth slice/scaffolds and implanted subcutaneously into immunodeficient mice, they differentiated into functional odontoblasts which generated the tubular dentine, as determined by tetracycline staining and confocal microscopy. These cells also differentiated into vascular endothelial cells, as determined by beta-galactosidase staining of LacZ-tagged SHED. The vascular endothelial growth factor (VEGF) induced SHED to express endothelium markers (VEGFR2, CD31, and VE-cadherin) and to organize the cells in vitro into capillary-like sprouts. Collectively, these experiments demonstrate that SHED can differentiate into endothelial cells that produce capillaries and odontoblasts capable of generating the tubular dentine, suggesting that the dentine matrix microenvironment plays a role in the fate of stem cells.

These basic areas of research involving cellular and molecular biology will provide guidelines for future translational experiments, targeting the regeneration of oral structures through the differentiation of stem cells of dental origin.

Scaffolds for Dental Pulp Stem Cells

The eukaryotic cells require interactions with their microenvironment to survive, proliferate, and maintain their activity. In tissue physiology, it is the extracellular matrix proteins which mainly form this "three-dimensional" environment. In tissue engineering, these "3D" structures are initially supplied to the cells through the use of biocompatible and biodegradable matrices, known as scaffolds [37]. They provide a favorable microenvironment for adhesion, migration, cell growth, and differentiation and allow the transport of nutrients, oxygen, and waste products. Therefore, the scaffolds are considered a critical component in tissue engineering [31, 38].

Depending on the purpose of application, the scaffolds can be synthetic or organic, biodegradable, or permanent [38, 39]. Matrices made of synthetic polymers allow the manipulation of their physicochemical properties, such as rate of degradation, pore size, and mechanical strength. Synthetic polymers most common in tissue engineering are the poly-L-lactic acid (PLLA), polyglycolic acid (PGA), and their copolymers such as poly-lactic-co-glycolic acid (PLGA). These scaffolds are biodegradable and biocompatible and allow cell growth and differentiation, making them highly suitable for applications in tissue engineering [40–42]. The rate of degradation can be controlled by the ratio of PLLA/PGA used in the manufacture of such matrices. Notably, it is important that the rate of degradation of the scaffold is compatible with the rate of formation of the tissue. In other words, the matrix must be programmed to ensure the structural integrity of the cells used in tissue engineering until the newly formed tissue can become self-sustaining [43].

One of the earliest successful examples of dental tissue engineering was the use of copolymers (PGA/PLLA and PLGA), which allowed the formation of complex tooth structures with characteristics similar to natural tooth crowns [42] Furthermore, the PLLA base scaffolds have been extensively used in the study of pulp tissue engineering [35, 36, 44]. These PLLA matrices are prepared inside the pulp chamber of tooth slices from extracted third molars. The dental pulp stem cells are seeded within the matrix/dentine slice and transplanted into the subcutaneous space of immunodeficient mice [35, 36, 45, 46].

Studies have shown that 21–28 days after transplantation, the dental pulp stem cells from the permanent (DPSCs) and deciduous (SHED) teeth seeded onto this model (polymeric matrix/dentine disk) produced tissue with morphological characteristics resembling those of natural human teeth pulp [45]. From the point of view of translational research, the matrices designed for dental pulp tissue engineering should be based on injectable materials. The purpose of these injectable scaffolds would allow stem cell transplantation to the fullest extent of the root canal and pulp chamber. An excellent example of this approach was recently described by Galler and colleagues [47]. In this case, the production of Self-assembling Multidomain Peptide hydrogels was characterized as biocompatible and injectable. Interestingly, with the addition of a specific cleavage site of matrix metalloproteinase-2 (MMP-2) and dendrimer cell adhesion (RGD), survival and cell motility have been improved in these hydrogels.

In summary, the development and characterization of these scaffolds are rapidly becoming extremely attractive in the new field of dental materials, and it is, therefore, an emerging area which will certainly play a critical role in translating the laboratory results for implementation approach-based cellular therapies and tissue engineering in dental treatment.

Blood Vessels and Tooth Tissue Regeneration

The establishment and maintenance of a vascular supplement is an essential requirement for the natural development of tissue. Vasculogenesis is defined as the formation of new blood vessels from differentiating endothelial cells derived from progenitor cells. The temporal and spatial regulation of vasculogenesis is required for embryogenesis, since the loss of a single allele of the gene causes embryonic death [48, 49].

Recently, it was observed that the stem cells of the pulp of the primary teeth have the potential to differentiate into vascular endothelial cells through a process similar to that of vasculogenesis. It has been shown that SHED differentiate into functional endothelial cells as they transport blood through the newly formed capillaries [36]. These surprising findings suggest that in the future the stem cell source of the dental pulp may be useful in the treatment of severe ischemic conditions such as myocardial infarction and cerebral ischemia.

Conceptually, angiogenesis is the process of the formation of new blood vessels from preexisting vasculature, making it therefore intrinsically different from the process of vasculogenesis. While vasculogenesis is extremely important in the early stages of embryonic development, angiogenesis allows remodeling of vascular networks, assuming an important role in postnatal physiological responses (e.g., in wound healing) and in pathological conditions.

In the context of the dental pulp, it is recognized that the events of healing of conservative treatments (direct pulp capping and pulpotomy) are orchestrated by a response regulated by angiogenesis. During the physiological process of healing, the sites of injured cells release chemotactic factors that contribute to the organization of the inflammatory process [50, 51].

Notably, the local cells release angiogenic factors that quickly organize an intense pro-angiogenic response, which allows for the influx of inflammatory cells and delivers oxygen and nutrients that are needed to maintain the high metabolic demands of cells actively engaged in tissue repair [50]. In dental pulp, a study showed that endothelial injury is involved in the recruitment of odontoblasts in the injury site [52].

Several bioactive molecules participate in inflammatory events and neo-formation of capillaries. Among them, vascular endothelial growth factor (VEGF) is considered the most important regulator of vasculogenesis and angiogenesis in physiological and pathological conditions [53, 54]. VEGF induces endothelial cells to form capillary structures, when cultured in collagen gel [55]. In vivo, VEGF increases permeability and induces potent pro-angiogenic responses [56, 57]. In addition, VEGF plays a critical role in the regulation of angiogenesis by increasing the survival of endothelial cells [55].

It has been observed that VEGF induces angiogenesis and improves survival of dental pulp cells of human tooth slices transplanted into the subcutaneous space of immunodeficient mice [58]. It has also been observed that VEGF induces the differentiation of SHED endothelial cells [36]. Iohara and colleagues [59] demonstrated that stem cells from porcine pulp increased blood flow in areas of experimental ischemia by the secretion of pro-angiogenic factors (VEGF), inducing an angiogenic response by the endothelial cells. It was also demonstrated that stem cell pulp under conditions of hypoxia, as occurs in some cases of dental trauma, induces pro-angiogenic responses directly related to the expression of VEGF by these cells [60]. Collectively, this data suggests that a local increase in bioavailability of VEGF is highly beneficial for the regeneration of complex dentine-pulp.

As mentioned earlier, the dentine matrix containing VEGF probably contributes to the angiogenic response mediated by dentine extracts [61]. Recently, the possibility of incorporating VEGF in scaffolds has been explored for use in dental tissue engineering. However, the biggest challenge yet for tissue regeneration is to ensure the rapid establishment of efficient anastomoses of blood vessels, facilitating the survival of transplanted cells and providing the flow of oxygen and nutrients needed to maintain the high metabolic demands of cells involved in tissue regeneration.

Isolation and Characterization of Stem Cells from Tooth Structures

To comprehend the stem cell characteristics of dental tissue, it is necessary to understand the developmental process of teeth. Tooth development is initiated by the interaction of mesenchyme and oral epithelium. However, teeth are derived from the oral ectoderm and neural crest-derived mesenchyme and are composed of six sequential steps, such as thickening, bud, cap, bell, secretory, and eruption.

The pulp is formed from the dental papilla; cementoblasts and periodontal tissues are formed from the peripheral dental follicle. The mature tooth is composed of the enamel, dentine, pulp, and periodontal ligament tissue. Deciduous teeth begin to erupt in humans at an average of 6 months after birth and the deciduous dentition is complete at 3 years of age. After 6 years of age, the dentition is already mixed with the presence of the first permanent teeth but yet with all the maintenance of the deciduous teeth. However, with the progression of facial development, all the deciduous teeth are substituted by the permanent successors [62, 63]. The last permanent tooth to erupt is the third molar. This generally occurs between the ages of 16 and 20.

The first type of dental stem cell was isolated from the human pulp tissue in 2000 from normal human impacted third molars. The dental pulp stem cells (DPSCs) were primarily derived from the pulp tissue of human impacted third

molars [17]. However, these cells were also isolated from supernumerary [64] and natal teeth [65]. Extracted human third molars represent an easily accessible, often discarded tissue. In 2003, stem cells from exfoliated deciduous teeth (SHED) were isolated and characterized (normal exfoliated human deciduous incisors) [18].

Subsequently, three more types of dental mesenchymal stem cell populations were isolated and characterized. In 2004, periodontal ligament stem cells (PDLSCs) were isolated from normal human impacted third molars [66]. The stem cells from the apical papilla (SCAP) were extracted from normal human impacted third molars [67, 68]. In 2005, Morsczeck and colleagues isolated precursor cells from human dental follicles of normal impacted third molars, called dental follicle progenitor cells (DFPCs), or dental sac, which is an ectomesenchymal tissue surrounding the developing tooth germ [69].

In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [70] established minimal criteria to define human MSCs, which include the following: (1) capacity of adhesion to plastic when maintained in standard culture conditions; (2) expression of CD105, CD73, and CD90 and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules; and (3) differentiation into osteoblasts, adipocytes, and chondroblasts in vitro.

Table 15.1 summarizes the methods of isolation, the in vivo and in vitro characterization, and the plasticity of the five types of dental stem cells.

Table 15.1 Human dental stem/progenitor cells (*hDS/PC*) from dental tissue: isolation, in vitro and in vivo characterization, and plasticity

hDS/PC from dental tissues	Isolation	In vitro characterization	In vivo characterization	Plasticity
DPSCs	Enzymatic digestion of pulp tissue after separating the crown from the root of normal human impacted third molars [17]	Cells showed clonogenic capacity and are highly proliferative and capable of regenerating tissue [17]	DPSCs were capable of differentiating into odontoblasts and adipocytes and expressing nestin and GFAP. They formed dentine-pulp-like complex after in vivo transplantation [71]	Osteo-/dentinogenic [68] and neurogenic differentiation [72]
SHED	Enzymatic digestion of pulp tissue after separating the remnant crown from normal exfoliated human deciduous incisors [18]	High proliferation rate and number of population doublings [18]. Cell morphology similar to fibroblasts and high viability of the cells in first passage. More than 90 % of cultured cells positive for MSC markers [73]	Dentinogenic and neurogenic differentiation in vivo [18]	Adipogenic [18, 73], neurogenic [18], chondrogenic, and osteogenic differentiation in vitro [73]
SCAP	Enzymatic digestion of the apical papilla after separation from the surface of the immature roots from normal human impacted third molars [67, 68]	Showed fibroblast-like morphology [74] positive for STRO-1, higher telomerase activity than DPSCs from the same tooth, and improved migration capacity in a scratch assay, compared to DPSCs from the same tooth [67]	Cells were positive for STRO-1 and CD146 markers [73]	Osteo-/dentinogenic [68], odontogenic, and adipogenic differentiation were obtained from SCAP cultures [74]
PDLSCs	Enzymatic digestion of periodontal ligament after gently separated from the surface of the root from normal human impacted third molars [66]	The ability of PDL-derived cells to form adherent clonogenic cell clusters of fibroblast-like cells, similar to those recorded for different mesenchymal stem cell populations. PDLSCs showed clonogenic capacity, highly proliferative cells, capable of regenerating cementum/PDL-like tissue, properties that effectively define them as stem cells [66]	Express MSC markers. When transplanted into immunocompromised rodents, PDLSCs showed the capacity to generate a cementum/PDL-like structure and contribute to periodontal tissue repair [66]. Ex vivo-expanded ovine PDLSCs exhibited a high proliferation rate in vitro and expressed a phenotype consistent with human-derived PDLSCs [75]	Capacity to develop into cementoblast-like cells, adipocytes in vitro, and cementum/PDL-like tissue in vivo. Contain a subpopulation of cells capable of differentiating into cementoblasts/cementocytes and collagen-forming cells in vivo [66], as well as cells with potential to differentiate into osteoblasts, chondrocytes, and adipocytes, in vitro [76]
DFPCs	Explant cultures or enzymatic digestion of attached dental follicles after separation from normal human impacted third molars [69]	These fibroblast-like, colony-forming, and plastic-adherent cells expressed putative stem cell markers Notch 1 and nestin [69]	Human DFPCs are capable of differentiating into periodontal tissues in vivo, expressing bone sialoprotein and osteocalcin after transplantation in immunocompromised mice, but without any sign of cementum or bone formation [69]	Dental follicle derived from human third molar teeth represents a rational source for precursor cells for PDL-FB, cementoblasts, and osteoblasts for alveolar bone [69]

DPSCs dental pulp stem cells, *SHED* stem cells from exfoliated deciduous teeth, *SCAP* stem cells from apical papilla, *PDLSCs* periodontal ligament stem cells, and *DFPCs* dental follicle progenitor cells

Regenerative Strategies and Clinical Use of Stem Cells from Dental Tissue

Bone Regeneration

Stem cells represent an easy and natural alternative to repair/regenerate damaged tissue, such as bone. This is essential especially when bone loss subsequent to degenerative or traumatic diseases cannot be amended through conventional therapies. Dental pulp stem/progenitor cells (DPCs) have been used for bone tissue engineering. d'Aquino and colleagues [77], in a clinical study, demonstrated that a DPC/collagen sponge biocomplex can completely restore human mandible bone defects and indicated that this cell population could be used for the repair and/or regeneration of tissue and organs. According to the authors, autologous DPCs are a new tool for bone tissue engineering.

DPSCs can also be used to improve the osteointegration of dental implants. According to Mangano and colleagues [78], titanium surfaces with stem cells from human dental pulp were capable of obtaining osteoblast differentiation of DPSCs and producing an appreciable amount of bone morphogenetic proteins as well as vascular endothelial growth factor and specific bone proteins. Therefore, complete osteointegration is obtained.

Different types of dental MSCs can be used in tissue engineering/regeneration protocols as an approachable stem cell source for osteo-/odontogenic differentiation and biomineralization that could be further applied for stem cell-based clinical therapies. According to Bakopoulou and colleagues [79], DPSCs and SCAP, both of which are types of MSCs, display an active potential for cellular migration, organization, and mineralization, producing 3D mineralized structures. These structures progressively expressed differentiation markers, including DSPP, BSP, OCN, and ALP, having the characteristics of osteodentine. The SCAP, however, show a significantly higher proliferation rate and mineralization potential, which might be of significance for their use in bone/dental tissue engineering.

The use of silk fibroin scaffolds combined with human stem cells, such as DPSCs and amniotic fluid stem cells (hAFSCs), has been successfully applied to repair critical-size cranial bone defects in immunocompromised rats. According to Riccio and colleagues [80], the progenitor cell association with an appropriate scaffold represents an optimal tool of bone regeneration and tissue engineering applications. It is a promising approach for the reconstruction of large human skeletal defects in craniofacial surgery.

Lesions of the Cornea

Gomes and colleagues [81] carried out a transplantation of tissue-engineered human undifferentiated immature dental pulp stem cell (hIDPSC) sheets in an animal model. This study was shown to be a valid alternative for ocular surface reconstruction in cases of bilateral total limbal stem cell deficiency and provides a new perspective in the field with important clinical implications.

Neurological Disease

Adult human DPSCs provide a readily accessible source of exogenous stem/precursor cells, which have the potential for use in cell therapeutic paradigms to treat neurological disease [71]. DPSCs reside within the perivascular niche of dental pulp and are thought to originate from migrating cranial neural crest (CNC) cells. During embryonic development, CNC cells differentiate into a wide variety of cell types, including neurons of the peripheral nervous system. Previously, it was demonstrated that DPSCs derived from adult human third molar teeth differentiate into cell types reminiscent of CNC embryonic ontology. According to Arthur and colleagues [71], DPSCs, when exposed to the appropriate environmental cues, would differentiate into functionally active neurons. The study demonstrated that ex vivo-expanded human adult DPSCs responded to neuronal inductive conditions both in vitro and in vivo. Human adult DPSCs acquired a neuronal morphology and expressed neuronal-specific markers at both the gene and protein levels. Culture-expanded DPSCs also exhibited the capacity to produce a sodium current consistent with functional neuronal cells when exposed to neuronal inductive media.

According to Völner and colleagues [82], human dental follicle cells (or the DFPCs) displayed characteristics of neural progenitor cells, and they are a promising alternative for new cell therapy approaches. The authors demonstrated the differentiation of these cells into neuron-like cells after a two-step strategy for neuronal differentiation, showing that these cells were neural precursors without potential for glial cell differentiation.

According to Sakai and colleagues [83], tooth-derived stem cells (SHED and DPSCs) may be an excellent and practical cellular resource for the treatment of spinal cord injury (SCI). Their study showed that human dental pulp stem cells exhibited neuroregenerative activities, such as the following: (a) they inhibited the SCI-induced apoptosis of neurons, astrocytes, and oligodendrocytes, which improved the preservation of neuronal filaments and myelin sheaths; (b) they promoted the regeneration of transected axons by directly inhibiting multiple axon growth inhibitors, including chondroitin sulfate proteoglycan and myelin-associated glycoprotein, via paracrine mechanisms; and (c) they replaced lost cells by differentiating into mature oligodendrocytes under the extreme conditions of SCI.

It has been shown that DPSCs are responsive to the surrounding microenvironment, surviving, migrating, and

differentiating accordingly into the appropriate cell types within the avian host neural tissue. These observations confirm the specificity of the neuronal differentiation response by adult human DPSCs as previously reported for SHED. Furthermore, it has been suggested that DPSCs and SHED are appropriate candidates for further evaluation as stem cell therapy-based treatments using animal models representative of neurological diseases and injury [71].

Hepatocytes

The study of Ishkitiev and colleagues [19] successfully showed the differentiation of dental pulp cells into hepatocyte-like cells, demonstrating that the stem/progenitor cells of the dental pulp have a hepatic potential and can be an important cell source for liver cell therapy.

The MSCs from the permanent and deciduous teeth pulp, in culture, produce specific hepatic proteins. These cells also have the potential for performing specific functions for hepatocytes, including the ability to store the production of glycogen and urea. The use of these cells for hepatic diseases could signal a new approach for treatment of patients in autologous use, in the near future.

Diabetes

It was demonstrated that DPSCs derived from deciduous teeth could be differentiated into pancreatic cell lineage and offer an unconventional and non-controversial source of human tissue that could be used for autologous stem cell therapy in diabetes without risk of rejection. The methods that have been developed could be transferred from bench to bedside for the treatment of children with type 1 diabetes. It has been suggested that banking of dental pulp stem from deciduous teeth should be considered for those patients at risk of developing maturity-onset type 2 diabetes [84].

Vasculogenesis as a Potential Treatment for Ischemic Disease

Using a study model of nude rats, DPSCs were able to repair infarcted myocardium. This capacity was associated with an increase in the number of vessels and a reduction in infarct size, probably because of the ability of the cells to secrete proangiogenic and antiapoptotic factors. The degree of cardiac repair observed was similar to that obtained with MSCs from the bone marrow. Therefore, this study extends the knowledge of DPSCs' therapeutical properties and provides a new source of stem cells for the treatment of ischemic diseases [85].

Conclusion

Human dental tissue is showing to be a very interesting source of stem or progenitor cells with the potential of differentiating into different cell types. Another advantage is the fact that it is a noninvasive and disposable source of cells. Although it is a source with a very low number of cells, the strong capacity of these cells to proliferate and their high plasticity is a focus of interest for their use in cellular therapy and regenerative medicine for research or clinical proposals. It should be noted that the association of these cells with scaffolds has also been studied as a strategy to regenerate bone or tooth loss after trauma or disease. Besides bone and tooth regeneration, these cells could also be an innovative choice for treating other diseases, focusing on regenerative medicine.

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Dental Stem Cells: Risk and Responsibilities

16

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Introduction

In early embryo development, the tooth tissues are originated from the neural crest anatomic site. Neural crest stem cells are considered embryonic-like stem cells, which are maintained under the control of Hox genes. Moreover, they are clonogenic cells and are able to differentiate into various cell types, such as odontoblasts, osteoblasts, chondrocytes, neurons, melanocytes, and the muscles [11]. Following the migration of neural crest stem cells during fetal development, the oral epithelium- and cranial crest-derived mesenchymal cells arise, which next form the dental follicle and dental pulp [4, 13–17].

Since 2000, different oral fetal and adult tissues were used to isolate dental stem cells (DSCs), among which are the dental pulp of permanent teeth [9], exfoliated deciduous (baby) teeth [11], periodontal ligament, apical papilla, dental follicle, and jaw periosteum [7]. Indeed, the dental pulp of deciduous teeth was appointed by different researches as a source rich in stem cells free from any ethical concerns, especially of easy disposable access and minimally invasive [11]. Different stem cell populations, such as mesenchymal and neuroepithelial or a mixed population of both, can be found in the dental pulp from deciduous teeth because of their neural crest origin following different methods of stem cell isolation and cultivation [19, 12]. Stem cells, which were isolated from adult third molar teeth, show similar but not equivalent properties [11]. Our group contributed in this field by isolating human immature dental pulp stem cells (hIDPSCs), which are a mixed population of mesenchymal stem cells (MSCs) and neuroepithelial stem cells (NESCs) [5], which express some of the embryonic stem cell markers [12]. We discovered using diverse animal models that hIDPSCs can be applied without any immunosuppression protocol [10]. Furthermore, breaking a paradigm about MSC, whose commitment is restricted to the mesoderm, we showed that these undifferentiated cells after transplantation into corneal injury in rabbits can differentiate correctly and produce normal corneal epithelium [8].

It is of common knowledge that the differentiation potential of stem cells depends on their anatomic localization as well as on proper niche, among other factors. The perivascular niche of dental stem cells has been described by Shi and Gronthos [21]. The monumental discovery of our group that this niche is not unique and the additional niches have been found in the dental pulp. Figure 16.1 demonstrates that DSC niches besides the perivascular ones (Fig. 16.1a, b) are also localized in nerve networks in the nerve plexus (Fig. 16.1c) as well as in the subodontoblast zone (Fig. 16.1d) and in cell-free and cell-rich zones (Fig. 16.1e, f). The existence of these niches is perfectly consistent with the neural crest origin of dental stem cells and isolation of a mixed population of NESCs and MSCs [11, 18].

We also suggested the method of isolation, freezing, and cultivation of hIDPSC, which allows their immediate transfer into clinical studies [18]. Taking into consideration that DSCs present a unique set of properties which is different from adult stem cells of other sources, this chapter will discuss the potential benefits as well as the risk and responsibilities which are involved in the isolation, manipulation, and transplantation of these cells.

Potential Benefits

Our knowledge about stem cells is growing every day; however; consistent data about their use in biomedicine and biotechnology are still lacking. Moreover, existing data seem to be a puzzle. We need to put together different pieces in a logical way in order to come up with the desired solution.

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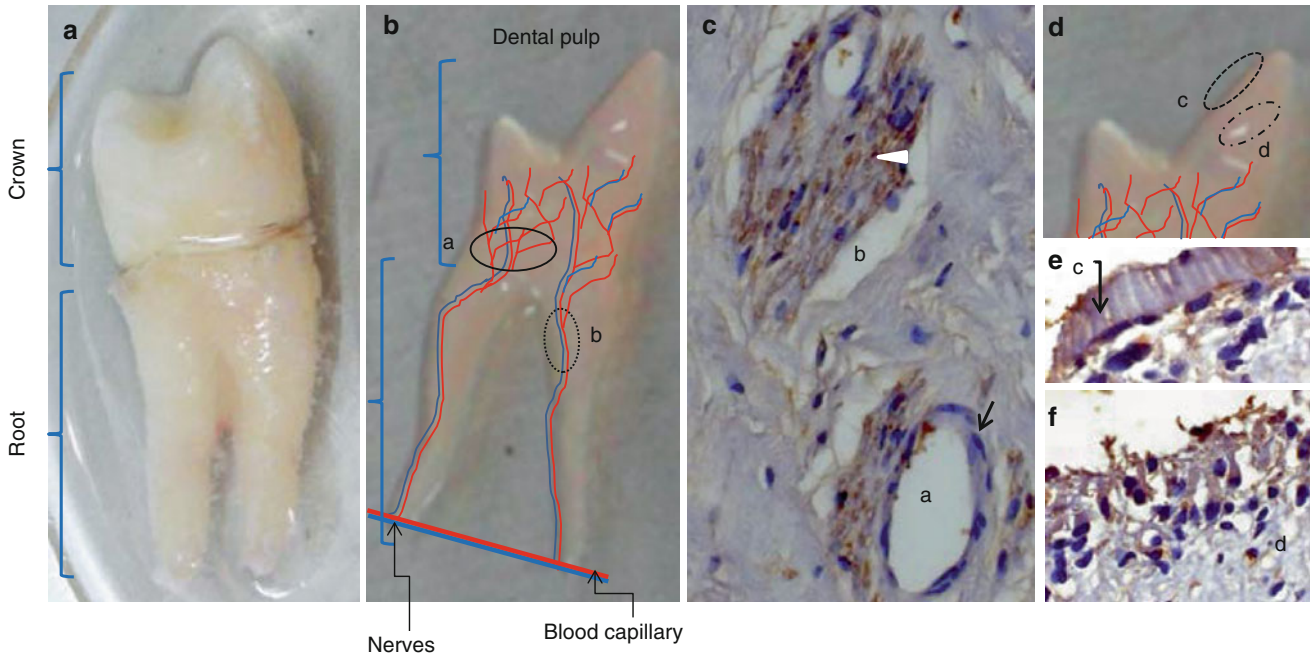

Fig. 16.1 Stem cell niches in dental pulp. (**a**) Teeth composed of crown and root (*blue*). (**b**) Dental pulp isolated from the teeth: (**a**) perivascular niche (*red*) and nerve plexus (*blue*). (**c**) Immunohistochemistry staining for nestin (early neuronal marker): negative reaction with perivascular niche (*black arrow*) (**a**) and positive in nerve plexus (*white arrowhead*) (**b**). (**d**) Dental pulp: (**c**) subodontoblast plexus niche and (**d**) cell-free and cell-rich zones' niches. (**e, f**) Immunostaining same as in (**c**)

The clinical use of stem cells may require the establishment of standards and creation of a particular order in stem cell isolation, manipulation, differentiation, and other procedures. Now, we are in the process of inquiry and discovery, which will allow us in the future to expand our knowledge and to understand what the stem cells are and how they should be applied into human organisms in order to provide the best regeneration process or even treatment of certain diseases.

The cells are organized into tissues, organs, and systems composing our organism. Stem cells can be found in all tissues of our organism and are responsible for homeostasis, thus providing a constant source for tissue self-regeneration in case of small injures, traumas, and diseases – anatomical sites of emergency (ASE). These cells are organized in stem cell niches, which are the main structures of stem cells and other cell types, which create a special microenvironment. These niches provide information to stem cells through the cascade of signaling molecules to move into a certain area of the body, when it is necessary. The stem cell niche is tightly controlled by the microenvironment that is responsible for everything from maintenance, proliferation, to eventual differentiation of stem cells. However, in case this microenvironment is damaged, stem cells will not be as effective as in a healthy environment. Once all these processes are under the control of the organism, it starts to recruit stem cells from other sources, which are nearby ASE or even from the more distant sources.

DSCs share their principal characteristics with bone marrow-derived MSCs (BM-MSCs), which were first discovered by Alexandre Friedenstein in the 1970s [6] and Arnold Caplan of Case Western Reserve University, who provided the MSC its current name, established a potential protocol for stem cell-based treatments, and conducted the first clinical trials in the United States [2]. DSCs are similar to BM-MSC, have immunomodulatory property, and do not trigger immune rejection of donated tissue or graft-versus-recipient disease, thus playing the role of a peacemaker when transplanted in ASE [1, 3, 8, 10].

DSCs demonstrate mechanisms of action similar to BM-MSCs, being able to support hematopoiesis, to provide anti-inflammatory and antiapoptotic effects, as well as to mobilize the patient's own stem cells in ASE. These paracrine mechanisms of action are beneficial for many diseases, for example, when apoptosis occurs in cells of the brain, which is caused by a hypoxic (oxygen-deprived) environment or depletion of blood cells.

The concept of stem cell niche provides comprehension of the complexity of the problem of therapeutic application of stem cells. This concept demonstrates that not only stem cells and their progenitors are involved in the tissue network but also a great number of different cell types as well as extracellular matrix. Therefore, the question is how one type of stem cells or progenitors will be able to regenerate completely and correctly the tissues whose formation occurred

during the life span. Indeed, depending on the type of injury or disease, one or more types of cells, such as stem cells, several progenitors, and differentiated cells, should be involved in regeneration. For example, the hematopoietic stem cell (HSC) niche is composed of different components such as osteoblasts and stromal cells, which are produced by the differentiation of MSCs in order to contribute positively to the microenvironment of HSC. The concept of adult stem cell niches leads us to the idea of multicell treatment; when different stem/progenitor cells with demonstrated potential to differentiate into some of derivatives of three embryonic germ layers should be used. DSC is the case; at least they demonstrate the capacity to differentiate into derivates of the mesoderm and ectoderm. These are considered as an alternate source of biological material for regenerative treatments, which use the cells, due to their origin and wide multipotential capacity.

We should consider the risk and responsibilities of any type of MSCs, particularly of DSC. Currently, very frequently in research laboratories, MSC isolation and application occur without checking for product quality. It is essential to increase caution to keep high-quality standards for cellular therapy products. Stem cells should be cultured under rigorous quality-controlled conditions, even for preclinical studies, in order to minimize the risk of any type of deviation of MSC therapeutic effect, as well as to balance the speed of clinical trials. Furthermore, before proceeding with clinical research on the transplantation of stem cells, several ethical criteria must be considered.

Risk and Responsibilities

Risk also refers to the possibility of harm or damage which could affect both the people and the community. The determination of current risks of stem cell isolation, manipulation, and transplantation is critical for the future of regenerative medicine. In order to meet research ethics committees' requirements, we need to evaluate the potential risks of MSC use and minimize such risks to society. This can be achieved through correctly designed preclinical studies on the transplantation of stem cells obtained under good laboratory practice conditions. Furthermore, clinical investigations in all the countries should be developed under the rigorous control of an international clinical research organization (CRO), which can validate these studies for the rest of the world, economizing the time and human efforts in order to achieve the clinical application of MSC in shorter time. So we avoid a current set of potential risks of stem cell transplantation in their further clinical application.

Responsibilities include confidentiality between the researcher and the donor/recipient during isolation, in vitro cultivation, expansion, as well as during preclinical and clinical studies. In the case that kids' biological material is involved in such investigation, the parents (first-degree relatives) should provide permission, as well as the kids should be informed in an understandable way about the aims of such studies. The researchers should be aware of their responsibilities and their commitment to safeguarding the safety and welfare of research subjects, featuring all that necessary for ensuring such.

Immune Rejection

Immunocompatibility is the most important concern for the clinical application of stem cells. The rejection of donor stem cells can interfere with the normal functions of the recipient immune system. To avoid such rejection, the immunosuppressive agents are used. However, various preclinical studies that used hIDPSC already reported the absence or a very low immunogenic response of the recipient organism [1, 3, 8, 10].

DSC showed immunogenicity and immunomodulatory properties similar to MSC, such as expression of intermediate levels of HLA class I and lack of expression of HLA class II and other costimulatory molecules like higher production of TGF-β and IL-10, resulting in immune evasion during transplantation. Moreover, these stem cells were shown to inhibit the proliferation of natural killer (NK) cells and T and B lymphocytes and impair dendritic cell maturation [11, 20, 22, 23].

Even if DSCs apparently are not rejected by the immune system, however, they may theoretically interfere with the normal functions of recipient immune response. Therefore, the understanding of their immunomodulatory properties has great relevance for DSC proper clinical use. The well tolerance of DSCs by the animal/human organism can also be attributed to the short-time survival of these cells in the recipient organism. On the other hand, if the survival of stem cells is prolonged in the recipient animal/human organism, the risk of immunogenic response against human/heterologous proteins produced by the cells should be regarded.

Tumor Formation

The risk of tumor formation after stem cell transplantation depends on the stem cell type, its proliferative and migration capacity, as well as on the site of injection. DSC when appropriately harvested and cultivated rarely may suffer malignant transformation, even after long-term in vitro culture. Several experiments using animals have shown that DSCs do not form tumors after transplantation [11]. To minimize the risk of tumor formation by these cells, several approaches can be considered, such as pre-differentiation of stem cells, shorter

time of stem cell in vitro cultivation, as well as the use of animal-free compounds. We recently provided a novel strategy of DSC isolation, manipulation, and cryopreservation, which leads to long-term culture of these cells with a limited number of DSC in vitro passages. Using this strategy, practically an unlimited amount of DSCs at early passages (before 5) can be produced [18].

Interestingly, it has been shown that adipose tissue-derived stem cells (ASCs) have affinity with the tumors in vivo, contributing to neovasculature formation, which feeds the growth of tumors. It seems that such affinity of ASCs correlates with their isolation from the perivascular niche [24]. Accordingly, DSCs can also be isolated from the perivascular niche. However, hIDPSCs are isolated also from distinct niches in the dental pulp, which is not a perivascular niche, thus minimizing the risk of tumor formation [18]. We should also consider the difficulty of the assessment of risks in preclinical studies on stem cell proliferation and tumor formation after transplantation once such events occur after a long time (more than the life spans of the animal models). This risk will be significantly higher if the stem cells will be transplanted into an organism which presents propensity to form the tumors.

Inappropriate Stem Cell Migration and Graft

Another eventual risk of DSC transplantation is the migration of stem cells from the graft site to inappropriate regions. Undifferentiated DSCs have an inherent ability to migrate to areas of inflammation and injury and participate in tissue repair. However, they have the capacity to undergo extensive migration from the site of injection to nontarget sites. Even if a low percentage of stem cells migrate and graft to other areas, this represents a theoretical risk of, for example, tumor formation.

Way of Application

The stem cell transplantation can be performed through different ways of application, such as systemic or local delivery, as well as, by transplantation of DSCs using tissue engineering approaches, such as the production of the cells in sheet or the cells that growing of scaffolds of different natures. However, any intervention to the recipient organism is potentially a site of local risk for infection, bleeding, and nerve damage. This risk will also vary depending on the way of stem cell transplantation. Even the size of stem cells is critical. The MSC of size above approximately 10 μm can potentially induce microvascular thrombosis, which can lead to inflammation and local hypoxia. So a single-cell suspension should be prepared for injection in order to avoid the aforementioned problem and eventual thrombosis or possible formation of local mineralized nodules by these cells.

Still, the success of transplantation has been limited owing to the problem of stem cell dosage. One of the obstacles of stem cell use in cell therapy, which is implicated in the successful development of stem cell technologies and their fast transference into clinical use, is the production of a great quantity of stem cells of excellent quality at early passages from the same donor [18]. Also, the clinical and pharmaceutical application of DSC is still hampered by a lack of tractable stem cell culture techniques. Good manufacturing practice can be a powerful tool to control the behavior and fate of stem cells that will be used in the design of new cellular therapies.

Infected Stem Cells

A risk associated with stem cell transplantation is the transmission of infectious agents from donor stem cells to the recipient organism. So several screening procedures are essential for guaranteeing a safe and trusty source of stem cells. The DSC can be contaminated by common oral pathogens, and all efforts should be applied, such as screening of the cells for pathogens, to reduce the risk of transmitting infectious agents from stem cells.

Patient Conditions

The patient conditions are crucial for the potential benefits of stem cell transplantation. To standardize the clinical application conditions for all patients is a difficult, but not impossible, task, and such success depends on the age of the patient and the severity of the symptoms of disease.

Final Considerations

Currently, the BM-MSC isolation occurs from volunteers, which are genetically distant (no parental relationship) from potential patients for cell therapy. The great advantage of DSC from deciduous teeth is their use by the parents, who share 50 %, or by grandparents, who share 25 % of the genetic background with the donor. However, the families that have a history of diseases, such as genetic, cancer, or other types, should avoid the use of stem cells from closely related family members. One of the possible risks of stem cell use obtained from unrelated individuals is a distribution of unknown genetic elements present in the human population, which can be minimized using stem cells from deciduous teeth in therapies.

Aging of stem cells in an organism is another problem that should be considered. DSC from deciduous teeth is an abundant source of young stem cells, which may benefit the oldest members of the family. Once stored, the stem cells from deciduous teeth can be constantly renewed within the family, thus avoiding a long-term cryopreservation [18].

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Stem Cells and Deciduous Teeth: Responsibilities

17

Vipin Arora and Pooja Arora

Introduction

Stem cell therapy has been used around the world to treat life-threatening conditions, and the full promise of stem cell therapy has only been glimpsed so far. Discoveries in stem cell research presents an opportunity for scientific evidences that stem cells, whether derived from adult tissues or the earliest cellular forms, hold great promise that go far beyond regenerative medicine.

Stem cells are a class of undifferentiated cells that are able to differentiate into specialized cell types [1]. According to the ability and potency to differentiate into different cellular types, three types of stem cells have been established: (1) totipotent stem cells (each cell has the capability of developing into an entire organism), (2) pluripotent stem cells (embryonic stem cells that are grown in vivo under induced conditions and are capable of differentiating into all types of tissue), and (3) multipotent stem cells (postnatal stem cells or adult stem cells with the capability of multilineage differentiation) [2].

Dentists are at the forefront of engaging their patients in potentially lifesaving therapies derived from their own stem cells located either in deciduous or permanent teeth. Postnatal stem cells have been isolated from various dental tissues [3]. So far, five types of dental stem cells have been identified: dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and dental follicle progenitor cells (DFPCs) [4–8]. Dental stem cells belong to the multipotent stem cell population [9].

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Recent studies have shown that SHED have the ability to develop into more types of body tissues than other types of stem cells [5, 10, 11], and there is an abundant source of adult stem cells in the human exfoliated deciduous teeth *(SHED)* [5]. Researchers have found the pulp of exfoliated deciduous teeth to contain chondrocytes, osteoblasts, adipocytes, and mesenchymal stem cells [5, 10]. All of these cell types hold enormous potential for the therapeutic treatment of neuronal degenerative disorders such as Alzheimer's, Parkinson's, and ALS (amyotrophic lateral sclerosis or Lou Gehrig's disease); chronic heart conditions such as congestive heart failure and chronic ischemic heart disease; and periodontal disease, to name a few, and to grow replacement teeth and bone [11–18]. One of the most important potential applications using these cells is for the treatment of paralysis due to spinal cord injury which has already been done using mesenchymal stem cells from other sources [15–17].

The application of stem cell therapy using SHED to treat these diseases is currently being pursued by many researchers at institutions around the world. There is much research left to be conducted, but the existing research has clearly shown that primary teeth are a better source for therapeutic stem cells than wisdom teeth and orthodontically extracted teeth [5]. Keeping this premise in mind, the concept of tooth banking has also popularized, and various companies have set up tooth banks to tap the potential of this new and innovative approach for preserving SHED and stem cells from other dental sources.

Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

While stem cells can be found in most tissues of the body, they are usually buried deep, are few in number, and are similar in appearance to surrounding cells. Until recently, stem cells harvested from umbilical cord blood were the only storage option to guard against future illness or disease. Unfortunately, the cord cell harvesting and storage process is beyond the reach of many people.

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With the documented discovery of SHED in 2003 by Dr. Songtao Shi, an accessible and available source of stem cells has been identified which can be easily preserved and used for future cure of ailments [5]. SHED are immature, unspecialized cells in the teeth that are able to grow into specialized cell types by a process known as "differentiation." SHED appear at the 6th week during the embryonic stage of human development. Scientists believe that these stem cells behave differently than postnatal (adult) stem cells [5]. SHED multiply rapidly and grow much faster than adult stem cells, suggesting that they are less mature, so they have the potential to develop into a wider variety of tissue types [5, 19]. Giordano et al. [20] stated that the dental pulp from adults as from deciduous teeth seems to be the most valuable form of stem cells due to the pluripotential type of cells.

Abbas et al. [21] investigated the possible neural crest origin of dental pulp stem cells from exfoliated deciduous teeth (SHED). Neural crest cells are multipotent cells that are capable of self-renewal and multilineage differentiation and play a major role in tooth development as they give rise to mesenchymal components of teeth including odontoblasts, pulp, apical vasculature, and periodontal ligament. They found that SHED are a heterogeneous population that shares common molecular characteristics with neural crest cells and stem cells in vitro. This ability to grow and regenerate tissues is the focus of the emerging field of personalized medicine which uses a patient's own stem cells for biologically compatible therapies and individually tailored treatments. Further, SHED are able to express proteins on their cell surfaces that allows them to differentiate not only into dental pulp, bone, and dentin but also into neural and fat cells (adipocytes) [5, 21]. In fact, SHED differentiate into nerve cells more readily than adult stem cells isolated from permanent teeth. SHED express a variety of neuronal and glial cell markers which directly reflect the embryonic neural crest origin of the dental pulp [5, 21]. SHED have been shown to express factors that induce bone formation and assist with the guidance of the eruption of the permanent teeth [5, 22]. Shi et al. [22] conducted a study on stem cells in adult human dental pulp (dental pulp stem cells (DPSCs)), human primary teeth (stem cells from human exfoliated deciduous teeth (SHED)), and periodontal ligament (periodontal ligament stem cells (PDLSCs)) by their capacity to generate clonogenic cell clusters in culture. Ex vivo expanded DPSC, SHED, and PDLSC populations expressed a heterogeneous assortment of markers associated with mesenchymal stem cell, dentin, bone, smooth muscle, neural tissue, and endothelium. Xenogeneic transplants containing HA/TCP with either DPSC or SHED generated donor-derived dentin pulp-like tissues with distinct odontoblast layers lining the mineralized dentin matrix. They concluded that the presence of distinct stem cell populations associated with dental structures have the potential to regenerate living human dental tissues in vivo.

Wang et al. [23] studied the characterization of stem cells from human exfoliated deciduous teeth (SHED) in comparison with dental pulp stem cells (DPSCs) to certify SHED as a key element in tissue engineering. SHED showed a higher proliferation rate and differentiation capability in comparison with DPSCs in vitro, and the results of the in vivo transplantation suggest that SHED have a higher capability of mineralization than the DPSCs.

Types of Stem Cells in Human Exfoliated Deciduous Teeth

Adipocytes

Adipocytes have successfully been used to repair damage to the heart muscle caused by severe heart attack. There is also preliminary data to indicate that they can be used to treat cardiovascular disease, spine and orthopedic conditions, congestive heart failure, and Crohn's disease and can be used in plastic surgery [14].

Chondrocytes and Osteoblasts

Chondrocytes and osteoblasts have successfully been used to grow bone and cartilage suitable for transplant. They have also been used to grow intact teeth in animals [5, 10, 11].

Mesenchymal

Mesenchymal stem cells have successfully been used to repair spinal cord injury and to restore feeling and movement in paralyzed human patients. Since they can form neuronal clusters, mesenchymal stem cells also have the potential to treat neuronal degenerative disorders such as Alzheimer's and Parkinson's diseases, cerebral palsy, as well as a host of other disorders [5, 15, 16, 22, 24]. Mesenchymal stem cells have more therapeutic potential than other types of adult stem cells [5, 17, 22].

Role of SHED in Craniofacial Tissue Engineering

Breakthrough lifesaving medical treatments using stem cells are being discovered and will continue to emerge in the decades to come. Koyama et al. [25] demonstrated that pluripotential cells isolated from the pulp of human teeth expanded in vitro and differentiated into osteoblasts, chondrocytes, and adipocytes. DPSC and SHED are not only derived from a very accessible tissue resource but also capable of providing enough cells for potential clinical applications.

SHED may be used to regenerate bone and correct craniofacial defects [10, 11]. Both in vitro studies and in vivo research in animal models have shown that tooth-derived adult stem cells can be used to regrow tooth roots in the presence of proper growth factors and a biologically compatible scaffold. Regenerative therapy is less invasive than surgical implantation, and early animal studies suggest comparable results in strength and function of the biological implant as compared to a traditional dental implant [17].

SHED are capable of extensive proliferation and multipotent differentiation, which makes them an important resource of stem cells for the regeneration and repair of craniofacial defects, tooth loss, and bone regeneration. Vakhrushev et al. [26] characterized cultures of multipotent mesenchymal stromal cells from the pulp of human deciduous teeth (SHED). The cells were used for population of 3D biodegradable polylactoglycolide scaffolds; their osteogenic potential was preserved under these conditions. Implantation of the scaffolds to mice induced no negative reactions in the recipients. These results suggested that the use of polylactoglycolide scaffolds populated with SHED is a promising approach for the creation of implants for bone defect replacement.

Zheng et al. [27] examined the efficacy of utilizing SHED in regenerating orofacial bone defects. The results indicated that stem cells from miniature pig deciduous teeth, an autologous and easily accessible stem cell source, were able to engraft and regenerate bone to repair critical-size mandibular defects at 6 months post-surgical reconstruction. In a similar study, Shen [28] evaluated the capacity of the stem cells derived from human exfoliated deciduous teeth in in vitro differentiation into osteoblasts. The purified CD34(+)/CD117(+) stem cells derived from exfoliated deciduous teeth of healthy children possess the capacity to differentiate into osteoblasts and form calcium deposits and mineralized nodules in vitro. Li et al. [29] stated that stem cells from human exfoliated deciduous teeth (SHED) are a unique postnatal stem cell population capable of regenerating mineralized tissue and treating immune disorders. However, the mechanism that controls SHED differentiation is not fully understood. Here, we showed that basic fibroblast growth factor (bFGF) treatment attenuated SHED-mediated mineralized tissue regeneration through activation of the extracellular signal-regulated kinase (ERK) 1 and 2 pathway.

SHED can be directly implanted into the pulp chamber of a severely injured tooth to regenerate the pulp inside the damaged tooth, preventing the need for endodontic treatment. Cordeiro et al. [30] evaluated morphologic characteristics of tissue formed when SHED seeded in biodegradable scaffolds prepared within human tooth slices were transplanted in immunodeficient mice. They observed that the resulting tissue presented architecture and cellularity closely resembling that of a physiologic pulp. Tissue-engineered bone grafts will be useful for practitioners in all of the dental specialties. Future applications may also include engineered joints and cranial sutures, which would be especially helpful to craniofacial and oral maxillofacial surgeons.

Hara et al. [31] focused on the characterization of SHED as compared with bone marrow-derived mesenchymal stem cells (BMMSCs). By using the gene expression profiles, this study indicated that SHED is involved in the BMP signaling pathway and suggests that BMP-4 might play a crucial role in this. These results might be useful for effective cell-based tissue regeneration, including that of bone, pulp, and dentin, by applying the characteristics of SHED.

In a study, De Mendonça Costa et al. [10] evaluated the capacity of human dental pulp stem cells (hDPSCs), isolated from primary teeth, to reconstruct large-sized cranial bone defects in non-immunosuppressed (NIS) rats. They found that hDPSC is an additional cell resource for correcting large cranial defects in rats and constitutes a promising model for the reconstruction of human large cranial defects in craniofacial surgery. Mao et al. [32] in a review discussed that adult stem cells have been isolated from the dental pulp, the deciduous tooth, and the periodontium. Several craniofacial structures such as the mandibular condyle, calvarial bone, cranial suture, and subcutaneous adipose tissue have been engineered from mesenchymal stem cells. They stated that craniofacial tissue engineering is likely to be realized in the foreseeable future and represents an opportunity that dentistry cannot afford to miss.

Potential Clinical Applications of Stem Cell Therapy with SHED

Stem cell-based therapies are being investigated for the treatment of many conditions, including neurodegenerative conditions such as Parkinson's disease and multiple sclerosis, liver disease, diabetes, cardiovascular disease, autoimmune diseases, musculoskeletal disorders, and for nerve regeneration following brain or spinal cord injury. Currently, patients are being treated using stem cells for bone fractures, cancer (bone marrow transplants), and spinal fusion surgery. New stem cell therapies are continually under review, and some have already been approved by the U.S. Food and Drug Administration. As the number of people affected by degenerative diseases continues to increase, there will be a greater need for new treatment options for the ever-growing aging population. Harvesting and banking SHED now will ensure their availability in the future when they will be needed most. This comprehensive list of diseases and conditions currently being treated using stem cells include stem cell disorders, acute and chronic leukemias, myeloproliferative disorders, myelodysplastic syndromes, lymphoproliferative disorders, inherited erythrocyte abnormalities, liposomal storage diseases, histiocytic disorders, phagocyte disorders, congenital immune system disorders, inherited platelet abnormalities, plasma cell disorders, and malignancies.

Given their ability to produce and secrete neurotrophic factors, SHED may also be beneficial for the treatment of neurodegenerative diseases and the repair of motor neurons following stroke or injury. Stem cells from third molars release chemicals that may allow the remaining nerves to survive the injury [15]. Future research will investigate if using tooth-derived stem cells can be done to regenerate neurons following spinal cord injury.

Inoue et al. [33] reported that dental pulp stem cells (DPSCs) ameliorated ischemic tissue injury in the rat brain and accelerated functional recovery after middle cerebral artery occlusion (MCAO). SHED-CM promoted the migration and differentiation of endogenous NPC, induced vasculogenesis, and ameliorated ischemic brain injury after pMCAO as well as DPSC transplantation. Taghipour et al. [34] assessed the potential in addition to neural-induced SHED (iSHED) for functional recovery when transplanted in a rat model for acute contused spinal cord injury (SCI). These findings have demonstrated that transplantation of SHED or its derivatives could be a suitable candidate for the treatment of SCI as well as other neuronal degenerative diseases. Karaöz et al. [35] stated that SHED not only differentiate into adipogenic, osteogenic, and chondrogenic lineage but also share some special characteristics of expressing some neural stem cell and epithelial markers. Under defined conditions, hDP-SCs are able to differentiate into both neural and vascular endothelial cells in vitro. Dental pulp might provide an alternative source for human MSCs.

hDP-SCs with a promising differentiation capacity could be easily isolated, and possible clinical use could be developed for neurodegenerative and oral diseases in the future. Nourbakhsh et al. [36] provided an evidence that SHED can differentiate into neural cells by the expression of a comprehensive set of genes and proteins that define neural-like cells in vitro. SHED might be considered as new candidates for the autologous transplantation of a wide variety of neurological diseases and neurotraumatic injuries. Razieh Alipour et al. [37] studied to examine and compare the expression of important stem cell surface markers on two populations of mesenchymal stem cells: one derived from human exfoliated deciduous teeth and the other derived from human adipose tissue. These new stem cells will offer a promising avenue for the prevention and reversal of many human diseases such as type 1 diabetes and prevention of liver fibrotic process.

Wang et al. [38] reported that SHED were able to differentiate into neural cells based on cellular morphology and the expression of early neuronal markers when cultured under neural inductive conditions. This study therefore investigated the therapeutic efficacy of SHED in alleviating Parkinson's disease (PD) in a rat model. They found that SHED could be induced to form neural-like spheres in a medium optimized for neural stem cells in vitro. Moreover, transplantation of SHED spheres into the striatum of parkinsonian rats partially improved the apomorphine-evoked rotation of behavorial disorders compared to transplantation of control SHED. Our data indicate that SHED, potentially derived from neural crest cells, may be an optimal source of postnatal stem cells for PD treatment.

Yamaza et al. [39] characterized mesenchymal stem cell properties of SHED in comparison to human bone marrow mesenchymal stem cells (BMMSCs). They utilized systemic SHED transplantation to treat systemic lupus erythematosus (SLE)-like MRL/lpr mice. They found that SHED are capable of differentiating into osteogenic and adipogenic cells, expressing mesenchymal surface molecules (STRO-1, CD146, SSEA4, CD73, CD105, and CD166), and activating multiple signaling pathways, including TGFbeta, ERK, Akt, Wnt, and PDGF. Recently, BMMSCs were shown to possess an immunomodulatory function that leads to successful therapies for immune diseases. They examined the immunomodulatory properties of SHED in comparison to BMMSCs and found that SHED had significant effects on inhibiting T helper 17 (Th17) cells in vitro. Moreover, they found that SHED transplantation is capable of effectively reversing SLE-associated disorders in MRL/lpr mice. At the cellular level, SHED transplantation elevated the ratio of regulatory T cells (Tregs) via Th17 cells. It was suggested that SHED are an accessible and feasible mesenchymal stem cell source for treating immune disorders like SLE.

Ueda and Nishino [40] investigated the interaction between stem cells from human exfoliated deciduous teeth (SHED)-derived growth factors and human dermal fibroblasts (HDFs) as the application of SHED for dermal wound healing remains speculative. Stem cells from human exfoliated deciduous teeth have effects on HDFs by increasing collagen synthesis and by activating the proliferation and migration activity of HDFs, suggesting that SHED or SHED-derived conditioned medium (SH-CM) can be used for the treatment of photoaging. They suggested that SHED and SH-CM should be constitutionally suited for photoaging treatment. Mainly with secreted growth factors or extracellular matrix proteins, SHED contribute to the enhanced wound-healing potential of HDFs.

Further mechanism studies using neutralizing antibodies against each growth factor may clarify the role of soluble factors of SHED in the wound-healing process. In a similar study, Nishino et al. [41] examined the effect on wound-healing promotion with unique stem cells from deciduous teeth as a medical waste. An excisional wound-splinting mouse model was used and the effect of wound healing among SHED, human mesenchymal stromal cells (hMSCs), human fibroblasts (hFibro), and a control (phosphate-buffered saline). SHED and hMSCs accelerated wound healing

compared with hFibro and the control. This implies that SHED might offer a unique stem cell resource and the possibility of novel cell therapies for wound healing in the future.

Limitations in Clinical Use

No doubt SHED have got multiple applications; there are certain limitations as well. The oncogenic potential of these cells is still to be determined in long-term clinical studies. Moreover, till date the research is mainly confined to animal models, and still human research trials are needed to document the same results in humans. Another main issue to consider is the difficulty to identify, isolate, purify, and grow these cells in the lab as these cells are required in large numbers to be therapeutically used. Immune rejection is also one of the issues which require a thorough consideration. Lastly, these are comparatively less potent than embryonic stem cells.

Advantages of Banking SHED

- It provides a guaranteed matching donor (autologous transplant) for life. There are many advantages of autologous transplant including no immune reaction and tissue rejection of the cells, no immunosuppressive therapy needed, and significantly reduced risk of communicable diseases.
- Saves cells before natural damage occurs.
- Simple and painless for both child and parent.
- Less than one third of the cost of cord blood storage.
- SHED are adult stem cells and are not the subject of the same ethical concerns as embryonic stem cells.
- SHED are complementary to stem cells from cord blood. While cord blood stem cells have proven valuable in the regeneration of blood cell types, SHED are able to regenerate solid tissue types that cord blood cannot – such as potentially repairing connective tissues, dental tissues, neuronal tissues, and bone.
- SHED may also be useful for close relatives of the donor such as grandparents, parents, uncles, and siblings.

Commercial Aspect of SHED Banking

These cells can be best utilized for the patients from which they are harvested and to a certain extent their immediate family and blood relatives. As such, it is inevitable that the key to successful stem cell therapy lies in being able to harvest the cells at the right point of development and to safely store them until accident or disease requires their usage. Needless to say, this means potentially storing for decades and the cost and technical difficulty of doing this properly make stem cell therapy using one's own cells a still uncertain bet. This is one aspect, but a strong lobby of researchers working with these cells considers banking of SHED as *biological insurance* and a ray of hope for the treatment of various ailments already discussed in the paper.

Till date, tooth banking is not very popular but the trend is catching up mainly in the developed countries. In the USA, BioEden (Austin, Texas) has international laboratories in the UK (serving Europe) and Thailand (serving Southeast Asia) with further expansion plans for Russia, Australia, India, and the Middle East. StemSave (USA) and Store-A-Tooth (USA) are also companies involved in banking tooth stem cells and expanding their horizon in other countries. In Japan, the first tooth bank was established in Hiroshima University, and the company was named as "Three Brackets" (Suri Buraketto) in 2005. Nagoya University (Kyodo, Japan) also came up with a tooth bank in 2007. Taipei Medical University (TMU) in collaboration with Hiroshima University opened the nation's first tooth bank in September 2008 with the goal of storing teeth for natural implants and providing a potential alternative source for harvesting and freezing stem cells including SHED. The Norwegian Tooth Bank set up in 2008 is collecting exfoliated primary teeth from 100,000 children in Norway. The tooth bank is a sub-project in the Norwegian Mother and Child Cohort Study (MoBa) and is a collaborative project between the Norwegian Institute of Public Health and the University of Bergen.

Summary

Stem cell therapy is emerging as a revolutionary treatment modality to treat diseases and injury, with wide-ranging medical benefits. SHED are stem cells found in the exfoliated deciduous/ primary teeth of children. Recent studies show that SHED appear to have the ability to develop into more types of body tissue than other types of stem cells. This difference opens the door to more therapeutic applications. There is much research left to be conducted, but the existing research has clearly shown that primary teeth are a better source for stem cells. While the promise of the immense scope and magnitude that stem cell therapies will have upon the population will only be fully realized in the future, dental professionals have realized that the critical time to act is now. The available opportunities to bank their patients' dental stem cells will have the greatest future impact if seized while patients are young and healthy.

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Breastmilk Stem Cells: Recent Advances and Future Prospects

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Summary

Optimally, breastmilk is the only food or drink that infants consume in the first 6 months of life. It is the optimal source of nutrition and protection for the human infant, containing nutritional agents and an array of bioactive factors that confer active immunity to the infant. As breastmilk composition is being researched, more and more factors are discovered in it with beneficial attributes. Interestingly, among its biochemical components, breastmilk contains maternal cells. Most of our knowledge on breastmilk cells comes from studies of leucocytic populations, which often are the dominant cells in colostrum and early lactation milk. However, in mature human milk, leucocytes are usually found in low numbers and the dominant cells are of epithelial origin. With the recent technical advancement in the fields of molecular biology and flow cytometry, the epithelial compartment of human milk has started to be further explored. These initial efforts are revealing a profound cellular hierarchy in breastmilk, from early-stage stem cells to progenitors to more differentiated lactocytes and myoepithelial cells. The origin(s) of these cell populations and their wide variability among and within women is also starting to be explored. Exciting advances are suggesting the existence of pluripotent stem cell populations in breastmilk, and this provides new avenues for examination of the role(s) of these cells in the lactating breast and for the breastfed infant and their potential uses in the study of the biology and pathology of the breast as well as in regenerative medicine. This chapter summarises the current knowledge of the cellular hierarchy of human milk and discusses future applications.

Introduction

Unique to the class Mammalia is the presence of mammary glands, specialised organs with the specific function of synthesis, secretion and delivery of milk to the newborn offspring upon demand during the lactation period [1]. Milk has the consistent role to provide optimal nourishment, protection and developmental programming to the young. Therefore, milk from different mammalian species varies in composition, reflecting the needs and growth rate of the young for which it is intended [2]. In addition to interspecies variations, milk composition varies within a species among different individuals and also within individuals, depending on the stage of lactation, infant feeding and maternal diet [3–9]. These variations add to the complexity of milk composition, which contributes to our lack of knowledge in this area.

Milk contains bioactive factors that confer immunological and developmental benefits [10]. Maternal cells have also been identified in milk, but their properties and role are not well established [1]. Most cellular studies of human milk have focused on colostrum (postpartum mammary secretion prior to secretory activation) or early lactation milk and its rich leucocyte content, which is implicated in the protection of the neonate against pathogens [11–13]. In humans, mature milk (breastmilk) contains fewer leucocytes and has higher levels of epithelial cells than colostrum [14–16]. More recent evidence shows that the leucocyte content of breastmilk rapidly decreases in women as breastmilk matures to reach a low baseline level (0–2 % of total milk cells) by the end of

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week 2 postpartum that is maintained throughout lactation unless the breastfeeding mother and/or infant have an infection [17].

Throughout most of the lactation period and under healthy conditions, nonimmune cells are clearly the most dominant cell type in human milk. Yet, there is little understanding of the nature, properties and developmental stages of nonimmune cells in breastmilk, particularly of inter- and intra-individual variations and factors influencing them. We have recently identified stem cells in breastmilk [18] and have commenced coordinated efforts to characterise them and explore their function(s) [19–23]. It is now understood that a cellular hierarchy is present in breastmilk, from early-stage stem cells with pluripotent features to multipotent and unipotent epithelial progenitors to differentiated lactocytes and myoepithelial cells [21]. Here, the current knowledge of this cellular hierarchy is presented, with special focus on the pluripotent stem cell populations of breastmilk, their properties and potential applications.

Cells in Human Milk: A Historical Account

Antonie van Leeuwenhoek first examined microscopic preparations of breastmilk particles [24], but it was not until Alexander Donné in 1837 that the presence of 'globules' (today known as milk fat globules) and 'granular bodies' (cells) in colostrum was confirmed with certainty [25]. Donné called these granular bodies 'corpuscles', without distinguishing their cellular identity. Henle [26] then corroborated these findings, substituting the term granular bodies for 'colostrum bodies'. In 1868, Beigel [27] confirmed the cellular nature of the granular/colostrum bodies described previously. Although some authors disputed the cellular nature of colostrum bodies describing them as agglomerations of fat globules [28], in subsequent years the cellular nature of the granular/colostrum bodies was confirmed by a number of reports. Bizzozero and Vassale [29] described them as mesenchymal cells, whilst Czerny [30] and Gruber [31] considered them leucocytes. Cregoire [32] and Engel [33] assigned them to the epithelial phenotype, whilst others described them as a mixed population of epithelium- and mesenchyme-derived cells [34, 35].

More than a century after the first recorded observation of cells in colostrum, Smith and Goldman [36] using more sophisticated methods confirmed that colostrum was rich in leucocytes. Before them, Holmquist and Papanikolaou [37] and others analysed smears of mammary secretion during lactation and other periods, aiming to develop a non-invasive method for the diagnosis and study of breast malignancy [38]. In summary, this early work suggested that the following cell types were present in colostrum and early milk:

1. *Foam cells* (*or 'cells of Donné'*), which have not been assigned further cellular identification. Holmquist and Papanikolaou [37] presumed that these cells were the 'colostrum or granular bodies' described by Donné and Henle. They report structural modifications of these cells that occur postpartum, including nuclear enlargement, increased frequency of multinucleated forms and greater cytoplasmic vacuolation. Later work suggests the foam cells to be leucocytes and/or lactocytes (milk secretory cells) as both the leucocytes and the foam cells stain with Sudan IV and oil red O, indicating the presence of lipid droplets within the cell.
2. *Leucocytes* were thought to be the most abundant blood cell type in smears of mammary secretions, particularly postpartum, mostly as single cells and less frequently as large aggregates.
3. *Histiocytes* were not considered to be 'characteristic of any one period' according to Holmquist and Papanikolaou [37]. Occasionally, they were found in large numbers, but no potential explanation for this was provided, such as correlation with inflammation.
4. *Epithelial cells* had been reported either single or in clusters in mammary secretions during pregnancy and the early postpartum period [37]. Holmquist and Papanikolaou classified them into five subtypes based on the cellular morphology, albeit highlighting the presence of transitional/intermediate forms [37]. Interestingly, each cluster type was assigned to a different epithelial differentiation stage, providing the first suggestion of a cellular hierarchy in breastmilk, involving undifferentiated cells.

The leucocyte component of colostrum and early lactation milk has been studied further with more advanced techniques; however, no in-depth investigation has been made of other cell types in breastmilk during different stages of lactation nor of factors associated with inter- and intra-individual variations. Exciting advances have been made in the last decade with the discovery of different epithelial cell types in breastmilk, including stem cells and progenitor cells [18, 19, 21].

Breastmilk Cells: Types, Properties and Origin

Human breastmilk contains a heterogeneous population of cells (Fig. 18.1a), which is variable both within a woman and between women. This variability is thought to relate to individual- and breast-specific factors as well as the stage of lactation. These factors potentially relate to the developmental stage of the mammary epithelium, the permeability of the basement membrane or infection, including maternal and infant disease, and may at least in part explain the wide variability in total cell counts and individual cell types per mL of colostrum and breastmilk reported in the literature. Despite this variability, it is established that colostrum and early lactation milk contain a larger proportion of leucocytes compared to mature breastmilk in humans and that the breastmilk epithelial cell content increases with lactation stage, becoming the predominant cell type in mature

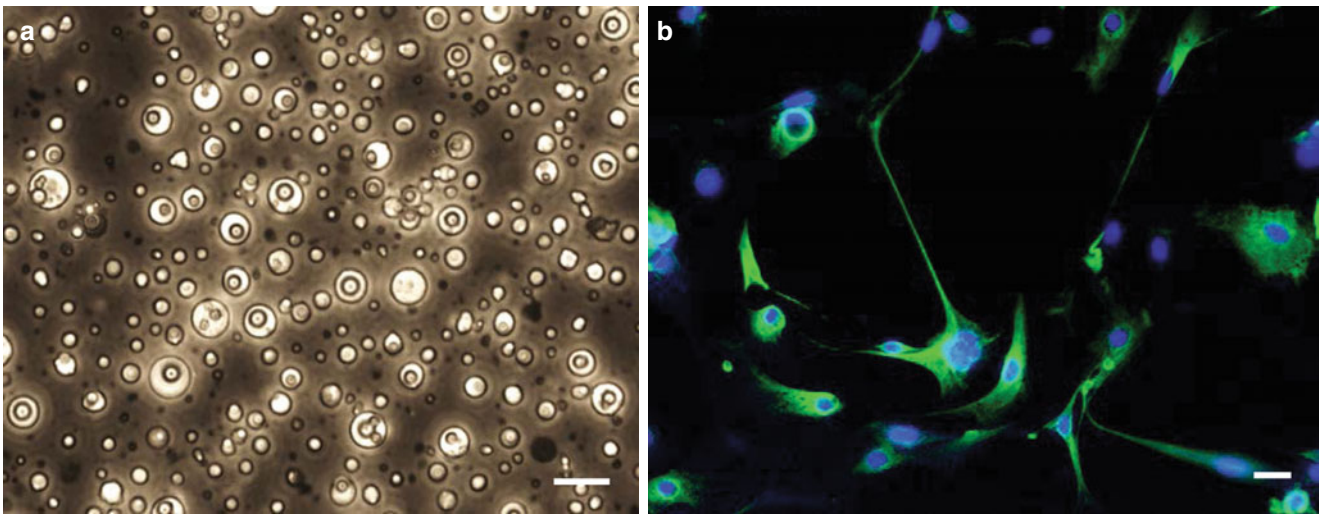

Fig. 18.1 (**a**) Freshly isolated breastmilk cells comprise a heterogeneous cell population. Scale bar: 10 μm. (**b**) Cells cultured from human breastmilk. *Blue*: DAPI (nuclear stain). *Green*: CK5 (cytokeratin 5). Scale bar: 50 μm

breastmilk from healthy mother-infant dyads [13–15, 17]. The total cell content of breastmilk also varies between and within women during lactation as well as during a single day, depending on the degree of breast fullness [39]. A range of 10,000–13,000,000 cells/mL breastmilk reflects the spectrum of interindividual variation observed to date, with colostrum and early lactation milk containing more cells than milk later in lactation [13, 39, 40]. Reported ranges of leucocyte content for colostrum and breastmilk are likely to be overestimated and the epithelial content underestimated when basic microscopic examinations are employed. This was highlighted by Engel [33] in 1953 when he pointed out the similarities between some milk leucocytes and epithelial cells, suggesting that misclassification of some cells may occur. The presence of lipid droplets in both leucocytic populations (e.g. macrophages) and lactocytes [11] partly explains this misclassification phenomenon. Thus, for reliable distinction between leucocytes and epithelial cells in milk, it is necessary to use techniques such as flow cytometry using monoclonal antibodies specific to unique surface markers. In this context, CD45 is of great value as it is a surface marker specific to leucocytes.

Leucocytes

Smith and Goldman [36] established the presence of leucocytes in breastmilk in 1966, and further studies have provided insight into the different milk leucocyte subpopulations and factors influencing them. Macrophages are the predominant leucocyte type in colostrum (40–50 % of total leucocytes), which also contains polymorphonuclear neutrophils (40–50 % of total leucocytes) and lymphocytes (5–10 % of total leucocytes) [11–13]. Of the lymphocyte populations, T cells constitute the majority (approximately 83 %) as opposed to B cells (4–6 %) [12]. Many of these leucocytes are activated, motile and interactive [36, 41]. Breastmilk leucocytes are thought to originate from the maternal blood circulation and be homed in the mammary gland under the control of hormones produced during lactation [12]. They are thought to be protective of breast infection and to respond to other maternal infections [17]. Recently, a response of breastmilk leucocytes has been demonstrated with not only maternal but also infant infections [17, 42]. The mechanisms of leucocyte movement into the breast and breastmilk in response to maternal and infant infections are still unclear.

Epithelial Cells

Epithelial cell populations are the predominant cell type in mature human breastmilk from healthy dyads [13, 17, 43]. This is in contrast to cow's milk, where the macrophage is the dominant cellular component throughout lactation [13, 43, 44]. Epithelial cells and cell clusters are common in breastmilk and can be isolated and cultured [45, 46], actively dividing at a rate depending on the sample and growth conditions, forming at least three distinct types of colonies: closed, open and mixed [47]. These have been thought to contain one or more of the main types of epithelial cells of the breast, the luminal cells (alveolar, $CK18^+$, and ductal, $CK19^+$) and the myoepithelial cells ($CK14^+/SMA^+$) and potentially more than one differentiation stages of these cell types. Brooker [13] performed a characterisation of the different epithelial cell types of breastmilk and reported three main types, which he identified as lactocytes (milk secretory cells), squamous epithelial cells and ductal cells. He found that in early lactation milk, the dominating epithelial subpopulations were of ductal origin, whereas as the milk matured the predominant cell type was the lactocyte, which contained fat globules.

These early studies suggested that breastmilk epithelial cells originate from different areas of the breast epithelium, both ductal and alveolar, and that their entry into breastmilk occurs via exfoliation from the epithelium due to the mechanical shear forces of breastfeeding [13, 37]. More recent evidence, however, suggests this epithelial detachment to be an active phenomenon associated with short-term changes in gene expression that increase cell motility [39].

Up until recently, breastmilk epithelial cells had not been identified at different differentiation stages. Immunofluorescence staining for stem cell and progenitor markers and flow-activated cell sorting (FACS) together with molecular techniques have allowed the identification in breastmilk of cells across a developmental hierarchy.

The Discovery of Stem Cells in Breastmilk

The mammary gland is unique in that it fully matures only during pregnancy and lactation and has the ability to undergo repeated cycles of proliferation, differentiation and apoptosis in response to hormonal signals that are initiated in gestation [1]. This ability to repeatedly undergo extensive remodelling has formed the basis for the postulation of the presence of stem cells and/or progenitor cells in this organ [46]. Recent studies using advanced in vitro and in vivo techniques have demonstrated the presence of bi-potent mammary stem cells (MaSCs) in the breast that display self-renewal and multipotency within the mammary lineage [48–51]. Cells with the phenotype $Lin^-CD49f^{hi}CD29^{hi}CD24^{+/low}$ are enriched in MaSCs; however, markers specific to the bi-potent MaSCs are not yet known. The cellular heterogeneity of the mammary gland adds an additional level of complexity to the delineation of the mammary cellular hierarchy across different developmental stages of this organ. The scarcity of specimens of normal human breast tissue biopsies, particularly during the lactation period, makes characterisation in women even more difficult. The use of breastmilk as a non-invasive, ethical and plentiful source of cells from the normal lactating gland overcomes this previously insurmountable issue [21].

The presence of an early stem cell and progenitor population in breastmilk is supported by reports of different epithelial morphological subtypes in breastmilk [13, 37, 52] and the speculation that each morphological type may represent a different developmental stage [37]. The ability to grow breastmilk cells in culture to form epithelial colonies that can be maintained through multiple passages [13, 45, 47, 52] further supports this theory. But it was not until 2007 when Cregan et al. provided the first evidence of breastmilk cells with stem cell and progenitor features [18]. Breastmilk-isolated cells were cultured in vitro forming colonies that contained cells expressing the stem cell markers CK5 (Fig. 18.1b) and nestin, among cells with the luminal ($CK18^+$) and the myoepithelial ($CK14^+$) phenotype [18]. Further studies have confirmed the presence of bi-potent MaSCs in breastmilk that express the stem cell genes p63 and CD49f, self-renew in spheroid culture and differentiate into $CK18^+$ and $CK14^+$ cells in culture under mammary differentiation conditions [19].

The high proliferation rates of MaSCs, reflected in the massive and rapid remodelling of the gland during pregnancy and lactation, prompted us to look for expression of genes that control self-renewal and potency. OCT4, SOX2 and NANOG, key genes controlling self-renewal and pluripotency in embryonic stem cells (ESCs) [53], were found to be expressed along with other ESC genes by breastmilk cell subpopulations, called human breastmilk stem cells (hBSCs) (Fig. 18.2) [21]. Expression levels vary widely between women and within a woman, and ongoing studies

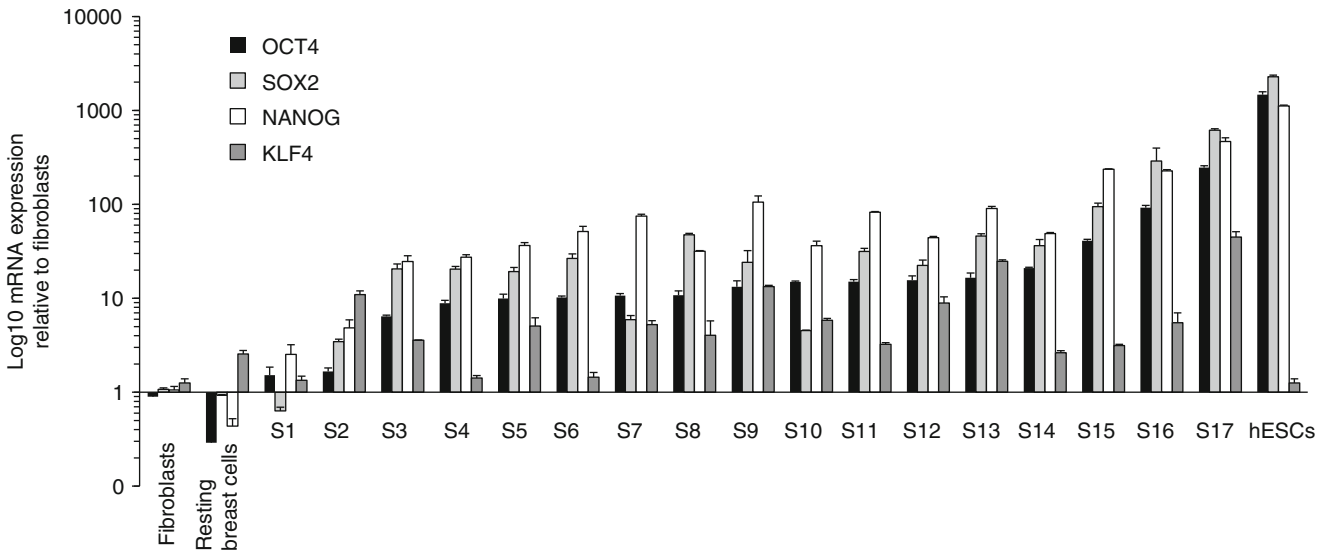

Fig. 18.2 Quantitative real-time PCR for expression of ESC genes by human breastmilk cells in 17 breastmilk samples (S1-S17) (Reproduced with permission from Hassiotou et al. [21])

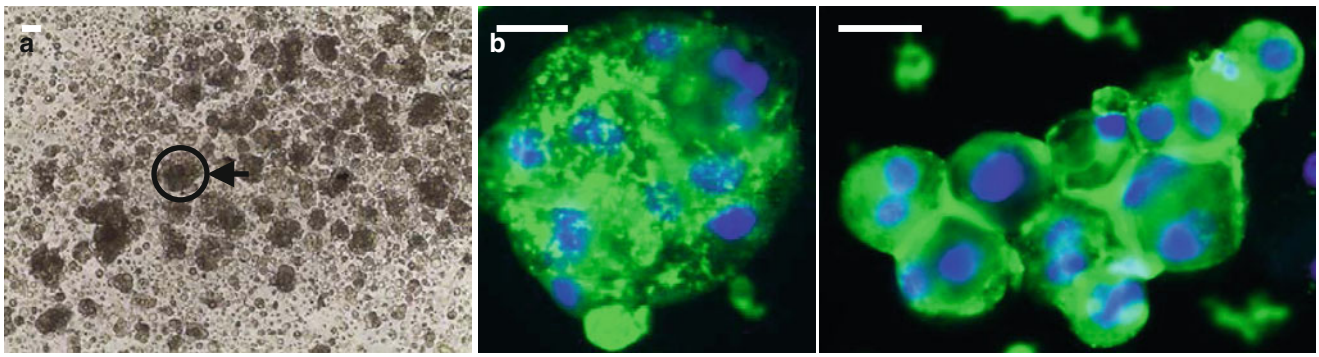

Fig. 18.3 (**a**) Spheroids cultured from human breastmilk stem cells. A single spheroid is highlighted in a *circle* with an *arrow*. Scale bar: 50 μm. (**b**) Production of milk proteins (*green*, β-casein; *blue*, DAPI, nuclear stain) by mammospheres generated from breastmilk cells under mammary differentiation conditions. Scale bars: 20 μm (Reproduced with permission from Hassiotou et al. [21])

are focusing on elucidating factors influencing this variability. Importantly, a method was identified by which successful expansion of these cells could be done in vitro in spheroid culture conditions (Fig. 18.3a).

When colony formation and clonogenicity was examined in hBSCs under MEF (mouse embryonic feeder fibroblasts) conditions in hESC growth medium and compared with hESCs, it was found that hBSCs formed ESC-like colonies that continued to express ESC genes after clonal expansion in secondary and tertiary feeder cultures [21]. In addition to self-renewal, the ESC transcription factor network comprising OCT4, SOX2, NANOG and their downstream targets controls pluripotency in ESCs [53]. Indeed, when we cultured hBSCs in various differentiation conditions, we observed differentiation outside the mammary lineage, both in a directed and spontaneous fashion [21]. hBSCs have now been shown to be able to differentiate in vitro into not only mammary alveolar-like units that secrete milk proteins (Fig. 18.3b) but also into cells with characteristics of neural cells, osteoblasts, chondrocytes, adipocytes, hepatocytes, pancreatic beta cells and cardiomyocytes (Fig. 18.4) [21]. It has been shown that although hBSCs display these similarities with human ESCs and induced pluripotent stem cells (iPSCs) ex vivo and in vitro, they do not form tumours when injected subcutaneously in SCID mice [21]. This suggests that hBSCs have controlled self-renewal properties, in contrast to ESCs and iPSC subpopulations, which often form tumours when injected subcutaneously in SCID mice.

In an effort to investigate the origin of the newly identified hBSCs with pluripotent features, we examined expression of ESC genes in rare human lactating breast tissue specimens. These genes were found to be expressed in the mammary epithelium [21] but also more scarcely by single cells in the mammary stroma (Fig. 18.5). In contrast, they were scarce in the resting mammary epithelium and in cultures of mammary cells isolated from resting breast mammoplasties [21]. Therefore, it is suggested that the cells observed in the lactating breast expressing pluripotency genes, and which can be accessed via breastmilk, are remnants from embryonic development and exist in scarce numbers in the resting breast, where expression of ESC genes is normally very low. The hormonal changes that occur during pregnancy and lactation stimulate upregulation of these genes in certain cell subpopulations, which start to actively divide in order to facilitate the mammary remodelling associated with the maturation of the gland into a milk secretory organ. Epigenetic mechanisms may be behind the regulation of ESC transcription factor expression in the breast, which may explain epigenetic activation of these genes in different stages of mammary gland development.

In addition to the above, the hypothesis of mobilisation of at least some of these cells from other organs and transfer to the breast through the blood circulation cannot be excluded and merits further investigation. Breastmilk contains a small population of cells expressing markers specific to haematopoietic stem cells, such as CD34 and CD133 [23], and it is currently unknown whether a proportion of these cells are also positive for ESC genes. Thus, the ESC-like hBSCs described by Hassiotou et al. [21] may be a heterogeneous population of stem/progenitor cells and/or not the only stem cell population present in breastmilk.

Further, the differential expression and subcellular localisation of ESC genes in breastmilk cells suggests the existence of different cell subpopulations within a sample, which may reflect different stages of cellular differentiation, from early-stage pluripotent stem cells to more committed progenitors to fully differentiated epithelial cells [1, 21]. This is further supported by the various colony types obtained from the culture of breastmilk cells, some of which do not display ESC features and may originate from more committed progenitor cells [21]. Thus, it becomes evident that a wide spectrum of developmental stages and functionalities is represented in breastmilk, reflecting the cellular hierarchy of the lactating mammary gland. Breastmilk therefore appears to be an excellent source of cells for the study of the biology and the pathology of this organ.

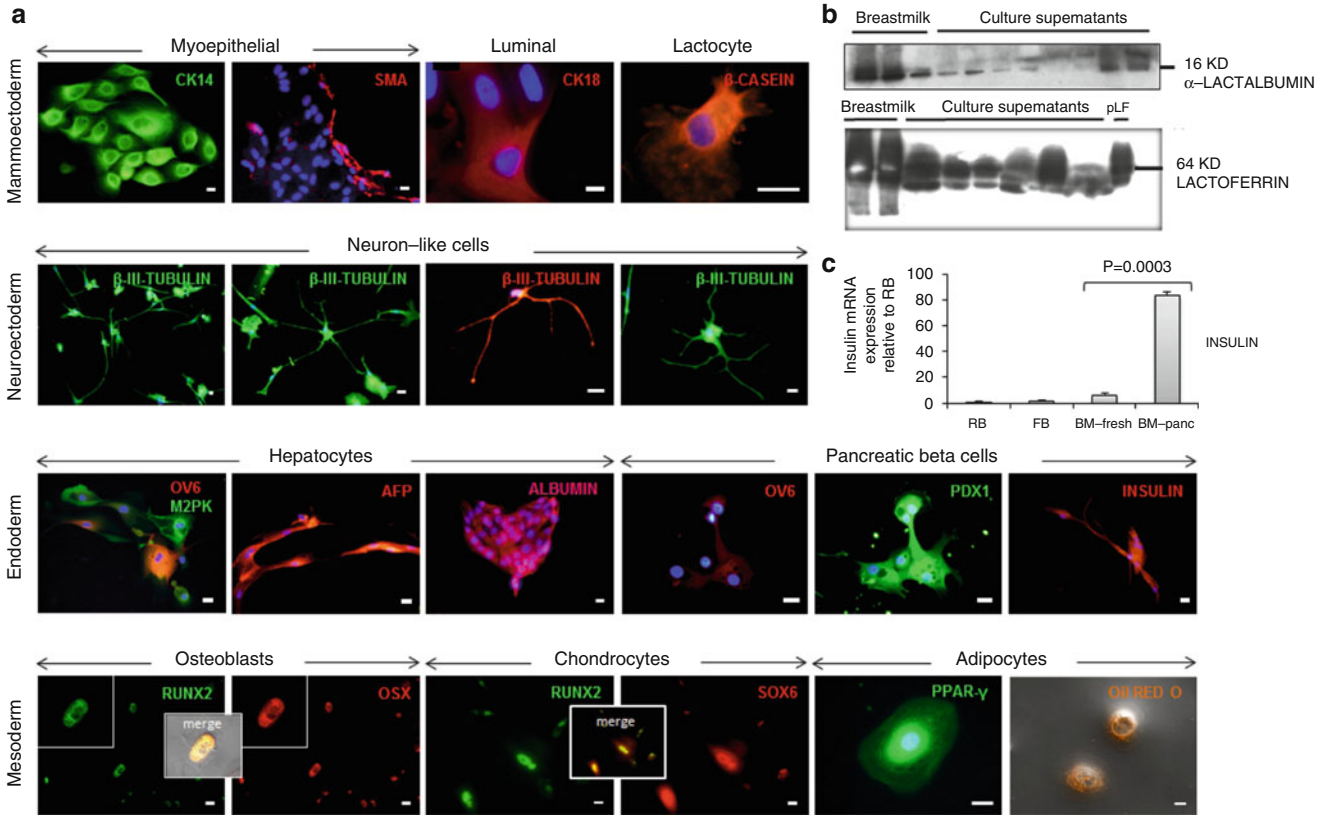

Fig. 18.4 Breastmilk stem cells differentiate into cells of the three germ layers. (**a**) Immunostaining for mammoectodermal, neuroectodermal, endodermal and mesodermal differentiation under corresponding growth conditions. *Blue*: DAPI (nuclear stain). Scale bars: 20 μm. (**b**) Western blot analysis of secreted milk proteins (α-lactalbumin and lactoferrin) in culture supernatants under mammary differentiation conditions. (**c**) Quantitative real-time PCR assay for insulin expression in breastmilk-derived cultures under pancreatic differentiation conditions. Insulin mRNA expression levels in freshly isolated breastmilk cells (BM-fresh) and breastmilk cells cultured under pancreatic conditions (BM-panc) are compared with those of mammary epithelial cells isolated from resting breast mammoplasties (*RB*) and human fibroblasts (*FB*) cultured under the same pancreatic differentiation conditions (Reproduced with permission from Hassiotou et al. [21])

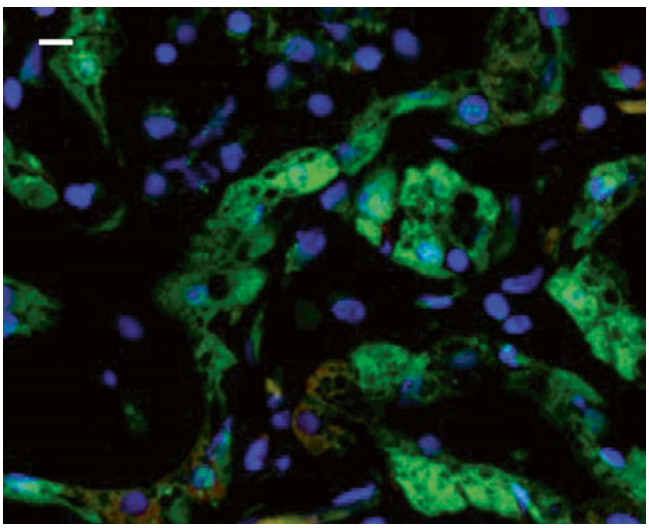

Fig. 18.5 OCT4 (*green*) expression in human lactating breast tissue. *Blue*: DAPI (nuclear staining). Scale bar: 10 μm

Functions of Breastmilk Stem Cells

It has now been established that at least a proportion of the stem cells found in breastmilk originates from the lactating epithelium. These cells are found in both the myoepithelial and luminal cell layers of the ductal and alveolar epithelium during lactation, with variation in the numbers of positive cells and their localisation between different parts of a duct and an alveolus and for different genes (Fig. 18.3) [1, 21]. The levels of gene expression vary not only between different cells of a duct and an alveolus but also between different lobules, suggesting different levels of differentiation and functionality between different lobules of a breast [1]. This is in agreement with previous studies showing differential expression of milk protein genes between different areas of the mammary epithelium in sheep and cattle [54], strongly suggesting a phenotypic and functional heterogeneity

between different epithelial compartments within a breast. This can also explain and is supported by the differences in milk production observed between the two breasts of a woman [5].

Thus, it becomes clear that the functional ability of a lactating breast and a lobule within a breast is associated with gene expression in MaSCs and the more differentiated cells in the epithelium. We propose that the numbers and properties of MaSCs expressing these newly identified ESC genes are critical for the physiological remodelling of the breast during pregnancy and lactation and may determine the course and level of differentiation of the mammary epithelium towards milk secretory tissue. Further research is necessary to confirm this hypothesis and examine the potential of gene expression in breastmilk-derived cells to be used as an indicator of breast tissue differentiation and functionality during lactation. This may lead to novel ways to assess and manage lactation difficulties, such as low milk supply, which is common particularly among mothers of preterm infants [55–57].

One of the most intriguing questions raised by the discovery of breastmilk stem cells with multi-lineage differentiation properties is their fate in and potential roles for the breastfed infant. Some of the early investigators of colostrum cells thought that these cells represented degenerating wandering cells in the lumen of alveoli [58, 59]. Today, it is clear that the great majority of breastmilk cells are viable at the time of milk expression [43], with breastmilk cell viability ranging from 70 to 100 % ($n=234$; >90 % for $n=202$) (Hassiotou, unpublished data); hence the infant consumes live cells during breastfeeding, some of which are stem cells. Given the cell content of breastmilk (10,000–13,000,000 cells/mL) [39] and normal daily milk consumption by infants (470–1,350 mL) [5], it can be estimated that breastfed infants ingest about 5×10^6 to 17×10^9 milk cells per day and 4×10^6 to 17×10^9 viable milk cells per day. It is not yet clear how many of these cells are stem cells and how many of the stem cells are capable of displaying pluripotential in vivo, but our studies so far suggest that this number changes during the day and at different stages of lactation. Assuming that the pluripotent stem cells in breastmilk have the phenotype $OCT4^+/SOX2^+/NANOG^+$, it can be estimated based on current data that this cell population appears in breastmilk in numbers of <1–30 % or more of total breastmilk cells (Hassiotou, unpublished data). Ongoing studies are examining the proportions of different phenotypes in breastmilk, associations with pluripotency and inter- and intra-individual variations.

The ingestion of such high numbers of stem cells by breastfed infants strongly suggests that these cells may have an active role in the infant. Although the viability and fate of breastmilk stem cells in the infant is not yet known, this has been studied for breastmilk leucocytes in animal models in an effort to understand whether they confer active immunity to the young. Some milk leucocytes survive the gastrointestinal tract of the young and resist digestion by trypsin [60]. In baboons, these cells have been shown to persist inside the gastrointestinal tract of the young either intraluminally or adhering to the gut epithelium for at least 60 h [61]. Based on these findings, it has been hypothesised that breastmilk leucocytes act to enhance the development of the intestinal mucosa. Furthermore, in a number of animal models such as neonatal baboons, lambs and rodents, some breastmilk leucocytes have been shown to cross the gut epithelium and enter the blood circulation, through which they are transferred to distant organs, including the mesenteric nodes, liver and spleen [61–65]. Therefore, it is highly likely that similar to breastmilk leucocytes, breastmilk stem cells may survive in the gastrointestinal tract of the breastfed infant, cross the digestive epithelium and enter the infant's systemic circulation, through which they are transferred to distant organs. Cell diapedesis through the gut epithelium may involve expression of chemokines and upregulation of adhesion molecules to allow cell binding and migration [66].

Entrance of live maternal breastmilk stem cells into the infant's systemic circulation may have numerous consequences. Transfer of these cells through the circulation into various organs may result in engraftment into these organs, contributing to tissue homeostasis, repair and/or regeneration, and thus optimal infant development early in life [21]. Indeed, such a function of breastmilk stem cells in the infant provides additional grounds for the existence of pluripotent stem cells in the lactating breast and breastmilk. This process of stem cell engraftment is called microchimerism and has been previously documented in the mother-offspring dyad. Stem cell exchange occurs in utero between the mother and the embryo and results in both embryonic cells being engrafted in maternal tissues and maternal cells being engrafted in embryonic tissues, where they can be detected viable for many years postpartum [67]. This process of stem cell exchange may continue postnatally via breastfeeding. The breastfed infant possesses a unique immunologic environment that is tolerised to maternal antigens and appears to not only tolerate the maternally derived breastmilk stem and other cells that it is exposed to but also potentially provide favourable conditions for their utilisation. Supportive of this is the better tolerance and success of maternal transplants in individuals that have been breastfed as infants [68, 69]. Future research should elucidate the role of breastmilk stem cells in the breastfed infant, which will allow evaluation of the importance of this newly identified breastmilk component in normal development. This will then provide new avenues for improvement of public guidelines for early infant nutrition, considering the absence of these cells from artificial milk.

In addition to human milk, preliminary evidence suggests that stem cells exist in the milk of other species (Hassiotou, unpublished). Indeed, the similarities of mammary gland anatomy and function between different mammalian species strongly support the presence of stem cells in milks from other species, which may end up there via mechanisms similar to human milk. Further research is needed to address this as well as the properties and potency of these cells. The presence of viable animal stem cells in milks that are consumed by humans, such as cow's milk, may create implications for human consumption of fresh milk from these animals.

Potential Applications of Breastmilk Stem Cells

Regenerative Medicine

The abundance of the cellular component of breastmilk together with its ethical, non-invasive and plentiful nature makes it an invaluable source of stem cells. For appropriate and safe use of these stem cells in regenerative medicine, it is prudent to first examine their proliferative capacities and potential of tumour formation when transplanted in vivo. The lack of any tumours in breastfed infants is a positive indication of the nontumorigenic character of hBSCs. In addition, a first attempt of subcutaneous transplantation of breastmilk fresh cells and breastmilk-derived hBSC spheroids into immunodeficient SCID mice did not demonstrate formation of any tumour [21]. Despite the common use of this assay as an indicator of in vivo pluripotency for hESCs and hiPSCs [70, 71], it is now well established that not all pluripotent cells form tumours when injected subcutaneously in mice [72–74]. And it is these nontumorigenic pluripotent cells that can be employed in regenerative medicine, providing they confer benefit(s) upon transplantation, whether this be integration in the transplanted organ and controlled division and differentiation into organ-specific functional cells, or signalling to host cells and stimulation of tissue repair and/or regeneration. Indeed, it has been shown that nontumorigenic pluripotent cells derived from the bone marrow integrate into damaged tissues when transplanted into immunodeficient mice by local or i.v. injection [72, 73]. Future work will examine this potential in hBSCs.

Therefore, the current evidence of the pluripotent and nontumorigenic character of hBSCs provides a first indication of their safety and potential usage in applications of regenerative medicine as a therapeutic alternative to hESCs and hiPSCs, the use of which is hindered by ethical and safety issues [70, 75, 76]. In particular, the functional in vitro differentiation of hBSCs into cells that produced insulin under pancreatic conditions or cells that produced albumin under hepatocytic conditions [21] shows great promise for the development of personalised treatments for diabetes and liver diseases. Further work on the hBSC capacity to differentiate into functional neurons may pave the way for innovative treatments for spinal cord injuries and neurodegenerative diseases, such as Parkinson disease.

Importantly, the tolerance of these cells by the breastfed infant provides an ideal immunologic environment for exploration of utilisation of hBSCs in the treatment of fatal neonate diseases, such as inborn errors in metabolism. It is of considerable interest that microchimerism has been documented between the mother and the offspring. Thus, a hBSC-based stem cell therapy for neonates with fatal diseases would only augment an existing physiological process. Perhaps the natural transfer of hBSCs to the infant via breastfeeding can be regarded as a natural stem cell therapy. This suggests that hBSCs may actually be more suitable for use in regenerative medicine than any other cell type currently used and/or explored for this purpose. This is because hBSCs are derived non-invasively and they potentially have the natural function of transferring into another organism and engrafting into it. Exploration of the potential therapeutic use of hBSCs in regenerative medicine will be aided by further studies of the ex vivo, in vitro and in vivo properties of these cells and their regulators.

Breastmilk Stem Cell Banking

In addition to extraction of stem cells from breastmilk for immediate therapeutic use, banking of these cells for their potential future use and/or for their use as donor cells for other individuals may also provide benefits. Umbilical cord blood stem cell banking has proven life-saving for many people, but the limited stem cell numbers that can be obtained from the cord and the high expense of their long-term storage pose limitations for their use. We have developed a method to expand hBSCs in culture and enhance their pluripotent properties [21]. This provides a basis for the development of breastmilk stem cell banking, which will be aided by further investigations of long-term maintenance of these cells.

Study of Breast Pathologies

The study of breast pathologies is another field in which the cellular population of breastmilk may provide insight. In the breast, complete cellular differentiation only commences during pregnancy and culminates during secretory activation in the early stages of lactation. Differentiation follows from controlled proliferation of the MaSC population that expands during pregnancy and lactation and continually remodels the breast until the cessation of lactation [1]. The pathways of MaSC proliferation and differentiation are currently poorly understood, but it is well established that failure in these

pathways is integral in the development of breast pathologies such as breast cancer and lactation difficulties.

Therefore, the cellular hierarchy present in breastmilk, which is thought to reflect that of the fully mature organ [1, 21], provides an excellent model for the study of breast pathologies in humans. It has been suggested that epigenetic modifications of the ESC-like cells identified in breastmilk maintain a controlled state of self-renewal in these cells that is characteristic and important for the normal function of the lactating breast [21]. The ESC transcription factor circuitry of OCT4/SOX2/NANOG that has been found to be expressed by these cells during lactation may be involved in maintaining this controlled state of self-renewal and allowing subsequent differentiation during the normal course of pregnancy and lactation. Disruption of this genetic network during pregnancy and lactation, failure to silence these genes during involution or the aberrant upregulation of these genes in the resting breast may all be factors associated with the initiation and/or progression of breast malignancy and its metastasis. In accordance with this model, it has recently been shown that forced ectopic expression of OCT4 in quiescent cells isolated from the resting mammary epithelium transforms these cells into rapidly dividing and highly tumorigenic cells [77]. The expression of these genes, albeit at differing levels, in both normal stem cells from the lactating epithelium [21] and cancer stem cells from breast tumours [78, 79] suggests a link between these two cell types and supports the use of breastmilk as a source of normal stem cells for the study of molecular determinants of breast cancer. At the same time, these breastmilk-derived cells offer novel opportunities to examine associations between gene expression in the breast and failure to produce milk during lactation or initiate copious milk production, particularly in mothers of preterm infants.

Concluding Remarks and Future Prospects

Breastmilk is considered to be a precious fluid both in developing and developed countries as it provides optimal nutrition, protection and developmental support to the infant. Most of the emphasis on the benefits of breastmilk has been attributed to its immunologic factors, both cellular and biochemical. Little attention has been given to other cell types of breastmilk, despite their dominance for the majority of the lactation period under normal conditions in humans. Recently, we identified stem cells in breastmilk, being present across different stages of lactation, a finding that gives a completely new dimension to the developmental benefits and importance of breastfeeding and breastmilk feeding. Most of these cells are viable in freshly expressed breastmilk, and breastfeeding results in ingestion of millions of these cells by the infant every day. This begs the question of the function of these cells in the breastfed infant. The ability of a subpopulation of these cells to differentiate into cells from all three germ layers in vitro and the appearance of other breastmilk cells in the systemic circulation of the young in animal models strongly suggest that a proportion of breastmilk stem cells are transported into different organs of the infant assisting in tissue homeostasis, repair and/or regeneration early in life. In addition to providing a new basis for investigation of the cellular benefits of breastmilk, hBSCs may be used therapeutically in regenerative medicine, a potential that should be further explored. Breastmilk also provides a non-invasive means of accessing cells from the human lactating breast, reflecting the physiological stage and functionality of this organ. Breastmilk therefore emerges as a new source of the cellular hierarchy of the fully mature breast, which may prove to be invaluable in future studies of the biology and pathologies of this organ.

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Part V

Animal Experimentation

Existence of Mesenchymal-Like Somatic Stem Cells in the Porcine Uterus

19

Katarzyna Miernik and Janusz Karasiński

Features of Adult Stem Cells

Adult stem cells are rare, undifferentiated cells residing in various tissues and organs within specific niches. They possess high proliferative potential and can persist throughout the lifetime of individual humans and animals. Self-renewal (maintenance of the undifferentiated cell population) is provided by asymmetric cell divisions. Such divisions also give rise to more differentiated progenitor daughter cells that are capable of rapid proliferation and differentiation (reviewed in [1]).

Adult stem cells are difficult to identify because they constitute a very small fraction of all cells and generally possess a few distinguishing surface markers. Alternative assays for the identification of adult stem cells rely on their functional properties [2]. Classically, adult stem cells were identified primarily by their clonogenic activity, defined as the ability of a single cell to produce a colony when seeded at extremely low densities. Other assays include examining self-renewal, ability to differentiate into one or more lineages, and in vivo tissue reconstitution [2, 3].

One of the specific stem cell types refers to the mesenchymal stem cells, which were found in various tissues and organs of human and animals, including pig bone marrow, peripheral blood, and adipose tissue [4]. Dominici et al. [5] proposed a set of minimal criteria defining mesenchymal stem cells (MSCs):

1. Adherence to plastic (in tissue culture).
2. Specific surface antigen expression (≥95 % of the MSC population must express CD105, CD73, and CD90, as measured by flow cytometry). Additionally, these cells must lack expression (≤2 % positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II.
3. Multipotent differentiation defined as the ability to specialize into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions.

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Anatomy and Physiology of Human Uterus

The main tissues of the adult uterus, the endometrium and myometrium, possess a remarkable capacity for remodeling and regeneration. At the cellular level, the adult endometrium consists of luminal and glandular epithelial cells, stromal cells, vascular smooth muscle cells, endothelial cells, and leukocytes. Anatomically and functionally, the human endometrium consists of the functionalis and basalis layers. The upper layer of the endometrium (functionalis) undergoes more than 400 cycles of proliferation, differentiation, and shedding during the woman's reproductive years [6, 7].

Within 48 h after shedding, the endometrial surface is rapidly covered by epithelial cells [1]. Then, it regenerates 4–7 mm of tissue due to intensive proliferation and differentiation of constituent cells. It is widely believed that the source of these proliferating cells is the basalis layer. Such remarkable regenerative capacity is equivalent to that of other highly regenerative organs such as the bone marrow, the epidermis, or the intestine [8]. Endometrial regeneration also follows parturition or extensive surgical resection and occurs in postmenopausal women taking estrogen replacement therapy [2, 9]. In non-menstruating species such as rodents and pigs, there are cycles of endometrial growth and apoptosis, rather than physical shedding of the functionalis layer. Remodeling of the endometrium (the stromal and epithelial cell proliferation and differentiation) is regulated by ovarian steroid hormones estrogen and progesterone in preparation for blastocyst implantation on a monthly basis in women, every 4–5 days in mice, and approximately every 21 days in pigs [10]. The uterus must rapidly enlarge to accommodate the developing fetus during pregnancy. This event comprises massive hypertrophy (an increase in cell number) and hyperplasia (an increase in cell size) of

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myometrial smooth muscle cells and other cell types and can be repeated multiply throughout the reproductive life of an individual [11].

Pig Model for Human Studies

For a long time, the mouse was the only animal model available for adult uterine stem cell studies [12, 17]. However, recently other animal models were used to investigate uterine stem cell biology, e.g., cow [13] and pig [10].

Casal and Haskins [14] argue that the mouse model may not be suitable enough to human study. Due to a short life span, longitudinal study on the murine model is not possible. Large animals (e.g., pig, cow, dog) are more similar to humans regarding life spans as well as organ size and genetic background. Therefore, according to Casal and Haskins, large animal models can be considered as "the translational bridge from in vitro and mouse experiments to human patients."

We postulate that due to structural and functional similarities between the human and porcine uterus and temporal physiological changes such as reproductive cycle duration, the pig may be a valuable candidate model for the investigation of uterine stem cells in normal and pathological conditions. However, to our knowledge, only one study is available so far in this scope [10].

Hypothesis on Adult Stem Cells in the Uterus

The hypothesis that the outstanding regenerative ability of the endometrium is associated with the existence of somatic stem cells in the uterus was formulated many years ago (reviewed in [2]).

Adult stem or progenitor cells residing in the endometrial basalis are considered to be responsible for cyclic regeneration of the functionalis compartment (reviewed in [2, 15]). It is also likely that myometrial stem/progenitor cells are involved in the dramatic enlargement of the pregnant uterus [7, 11].

Since the first evidence for the existence of clonogenic cells in the human endometrium was provided by Chan et al. [1], several studies have reported the presence of a variety of stemlike cells in both human and mice uteri [6, 9, 12, 16–19]. Hence, we aimed to find out whether the pig uterus also contains a population of adult stem-like cells by demonstrating clonogenicity, in vitro multi-differentiation capacity, and expression of stem cell markers [10].

Several recent studies strongly suggest that endometrial stem/progenitor cells are present in menstrual blood [15, 20, 21]. Indeed, the menstrual blood is derived from the shedding of the endometrial lining and contains fragments of the detached endometrium [22, 23]. Moreover, Musina et al. [23] showed that morphology of stromal cells isolated from menstrual blood cultured in vitro resemble bone marrow-derived MSC. It was demonstrated recently that menstrual blood-derived mesenchymal cells could serve as a potential novel source for stem cell-based therapy, e.g., for cardiac repair [20].

Clonogenicity

Chan et al. [1] provided initial evidence that the human endometrium contains epithelial and stromal cells with high proliferative potential. Approximately 0.22 % of the epithelial and 1.25 % of the stromal cell populations freshly isolated after hysterectomy were able to initiate large colonies when seeded at clonal density [1]. The cloning efficiencies for cells derived from other tissues varies between 0.01 % for colon and about 5 % for epithelial cells from the epidermis, prostate, and mammary gland [1]. Clonally derived epithelial and stromal cells from the human uterus are capable of self-renewal and differentiation into four lineages (osteogenic, chondrogenic, myogenic, adipogenic) and possess a high proliferative potential in culture [6].

Moreover, Schwab et al. [24] demonstrated that the clonogenicity of epithelial and stromal cells did not vary between the proliferative, secretory, and inactive human endometrium. This suggests that ovarian steroid hormones do not maintain the clonogenic potential of these cells. On the other hand, other studies indicate that the proportion of side-population cells (also regarded as local stem-like cells) isolated from the human endometrium varies with the phase of the menstrual cycle. The highest proportion of side-population cells was observed in the early proliferative phase [25] or shortly after menstruation [19, 26].

We have reported that 0.035 % of all cells isolated from the whole porcine uterine sample formed large clones consisting of more than 50 cells when seeded at low density [10]. Hence, a small population of highly clonogenic cells resides in the porcine uterus. In the porcine model, we did not observe a major difference between the clonogenicity of cells isolated from the uteri at preovulatory or secretory phases of the estrus cycle.

Stem Cell Marker Expression: Mesenchymal vs Hematopoietic

Uterine stem cells are generally positive for mesenchymal and negative for hematopoietic stem cell markers [6, 18, 27]. These mesenchymal stem cells seem to have properties and phenotype similar to bone marrow or adipose tissue SC [3]. However, some studies reported the presence of CD34 and CD45 hematopoietic markers on cells in human

endometrium [28] and showed they have vasculogenic potential in murine uterus [29].

Porcine uterine cells demonstrated the presence of mesenchymal stem cell markers CD29, CD44, CD144, CD105, and CD140b and the lack of hematopoietic stem cell markers CD34 and CD45 [10]. We have also shown for the first time that the uterine cells express an intermediate filament protein nestin, which is characteristic for immature neurons and other undifferentiated cells [30]. This protein was also found in adipose-derived stem cells [31].

The transcription factor Oct-4 (POU5F1) is involved in the self-renewal of undifferentiated pluripotent stem cells [32] and is frequently used as a reliable marker for undifferentiated cells. This protein is expressed in the human endometrium [33] and does not change during the menstrual cycle. This suggests that hormone-induced cyclic changes of the endometrium do not influence the expression of Oct-4 [34]. We have failed to detect the presence of Oct-4 in primary cell culture harvested from the porcine uterus [10]. Interestingly, Ono et al. [35] reported that the expression of Oct-4 decreases during in vitro culture and the mRNA level became undetectable after 14 days of culture.

Multipotent Differentiation

A functional property of mesenchymal stem cells is the ability to differentiate into adipocytes, osteocytes, chondrocytes, and myocytes when cultured in vitro in inducing media [3, 36–38]. Endometrial-derived cells with substantial clonal ability were shown to differentiate into the abovementioned four mesodermal lineages when cultured under appropriate conditions [6, 39]. It was also shown that endometrial-derived cells could differentiate into chondrocyte lineage, which was demonstrated by the expression of articular cartilage markers sulfated glycosaminoglycans and type II collagen [9]. Moreover, Wolff et al. [9] demonstrated that nonendometrial reproductive tissues (myometrial, fibroid, fallopian tube, uterosacral ligament tissue) lack multipotent differentiation ability as chondrocytes were not able to form in tissue culture. Other experiments indicate that human endometrial cells differentiate into adipocytes [18] or dopaminergic neurons [27]. One study so far presents that human myometrial progenitor cells possess the ability to differentiate into two lineages: osteogenic and adipogenic [11].

Interestingly, Donofrio et al. [13] demonstrated on bovine model that stromal endometrial cells revealed induced potential for osteogenic lineage. Moreover, during long-term culture, those cells spontaneously adopted a mesenchymal phenotype similar to bone marrow-derived cells and seemed to differentiate along the osteogenic lineage.

Multipotent differentiation was also proved for cells derived from the porcine uterus as isolated cells cultured in induced media were capable to differentiate into osteogenic and adipogenic lineages [10].

Other Methods to Analyze Adult Stem Cells

Side Population

In 1996, Goodell et al. identified somatic stem cells in murine bone marrow using fluorescent dye Hoechst 33342 [40]. Putative stem cell could rapidly efflux Hoechst, whereas other cells remained labeled. Therefore, FACS (fluorescent activated cell sorter) analysis records a distinct population of unlabelled cells, so-called side-population (SP) cells. Molecular studies revealed that the SP phenotype is connected with high-level expression of the ATP-binding cassette transporter protein ABCG2/Bcrp1 which is responsible for the removal of toxins from the cell [41]. The detection of SP enables to prospectively isolate stem cells from tissues with unidentified stem cell markers. Since 1996, SP cells were found in numerous tissues and organs of human and animal origin and are intensively studied in order to confirm their stem cell properties.

SP cells were isolated from the endometrium [19] and myometrium [11], confirming the existence of stem-like cell population in both layers of the uterus. Endometrial SP cells were primary CD9 and CD13 negative and, after longtime in vitro culture, formed colonies with glandular (CD9 positive)- or stromal (CD13 positive)-like structure. Cells were maintained in vitro for more than 24 weeks without senescence. Stem-like properties of SP cells isolated from human endometrium were then confirmed in further studies [16, 25, 26]. Myometrial SP cells lack or underexpress the myometrial cell markers, reside in quiescence, and, after transplantation into immunodeficient mice, produce functional human myometrial tissue. Under the appropriate differentiation-inducing conditions, myometrial SP cells differentiate into osteocytes and adipocytes. So far, pig uterus was not investigated regarding the presence of side-population cells.

Origin of Stem Cells in the Uterus: Potential Sources

According to Gargett [2], two main hypotheses could explain the origin of adult stem cells in the uterus: (1) remnant fetal stem cells and (2) recruited circulating stem cells from bone marrow.

The latter hypothesis is supported by several studies suggesting the contribution of bone marrow-derived cells to uterine regeneration in bone marrow recipients [42, 43]). BM-derived cells have the ability to differentiate into cells of mesodermal, ectodermal, and endodermal origin in BM

recipients. Bone marrow stem cells are shown to migrate to the sites of tissue damage, incorporate into various organs, and contribute to angiogenesis. Eventually, they may transdifferentiate into cells specific for the organ in which they reside (reviewed in [15]). In several studies, bone marrow-derived cells that are histologically and immunohistochemically similar to epithelial and stromal cells have been detected in the endometria of BM recipients (reviewed in [44]). However, in a recent paper, Cervello argues that bone marrow-derived cells migrating to the uterus after BM transplantation can represent only a limited source of transdifferentiated endometrial cells rather than a viable source for cyclical regeneration [44]. Thus, additional evidence is needed to draw definite conclusions regarding the origin of uterine stem cells.

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Human Menstrual Blood-Derived Stem Cell Transplantation for Acute Hind Limb Ischemia Treatment in Mouse Models

20

Ngoc Bich Vu, Van Ngoc-Le Trinh, Lan Thi Phi, Ngoc Kim Phan, and Phuc Van Pham

Introduction

Ischemia is conditionally caused by limited blood supply to tissues, which results in a shortage of oxygen and nutrients needed for cellular activities [38]. The main reason for ischemia is damage to blood vessels. Acute hind limb ischemia (ALI) is a medical condition caused by a sudden lack of blood flow to hind limbs [57]. ALI is due to either an embolism or thrombosis. Thus, ALI can be caused by peripheral vascular disease, air, trauma, fat, amniotic fluid, or a tumor [23]. In the United States, the rate of ALI is 14 out of every 100,000 people per year [13], in which 85 % of cases are due to arterial thrombosis and 15 % from embolic occlusion. Prolonged or delayed treatment of ALI can result in morbidity, amputation, and/or death. Therefore, ALI has been researched and treated for some decades. At present, almost all therapies for treating ALI are based on thrombolysis. There are three methods used to remove the thrombosis. In the past, doctors generally used streptokinase, but recently other factors have been developed for this purpose, such as tissue plasminogen activator, urokinase, and anistreplase. These factors are delivered into the ischemic region by pulsed spray catheters [3, 6]. At the thrombotic clot, such factors can lyse the thrombus [58]. The second method for clot lysis is using either saline jets or ultrasonic waves. Saline jets can dislodge the clot using the Bernoulli effect, whereas ultrasonic waves can create physical fragmentation of the thrombus [34]. The third method is creation of a "bypass" around the clot by inserting a graft [5].

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In recent years, stem cell therapy has been considered as another method to treat ALI. Compared with the three previous methods to treat ALI, stem cell therapy promises many advantages. In fact, stem cells can create new blood vessels that supply oxygen and nutrients to the ischemic area. Stem cells can be obtained from two main sources, namely, embryos and adult tissues. Embryonic stem cells have limitations because of teratoma formation and ethical dilemmas, whereas adult stem cells require invasive techniques to collect. Stem cells derived from menstrual blood is a new option to isolate stem cells for regenerative medicine. In humans, the endometrium has a remarkable regenerative capacity. For every menstrual cycle, it grows from 0.5–1 to 5–17 mm after the menstrual cycle, indicating that the endometrium is created by cyclic processes of cellular proliferation, differentiation, and shedding [36]. From 2005, many researchers have identified and isolated a cell population from menstrual blood and found that they show the characteristics of stem cells, particularly mesenchymal stem cells (MSCs) [14, 52, 53, 60], which have been called menstrual blood-derived stem cells (MenSCs). MenSCs express typical MSC markers such as CD29, CD44, CD49f, CD90, CD105, and CD166 and markers of embryonic stem cells such as Oct-4, SSEA-4, Nanog, and c-kit [2, 4, 42, 43, 63]. MenSCs have the ability to differentiate into various cell types derived from the mesoderm and endoderm, such as adipocytes, osteoblasts, chondrocytes, neurons, endotheliocytes, pulmonary epithelial cells, hepatocytes, islet cells, cardiomyocytes, and insulin-producing cells [21, 32, 37, 42].

MenSCs are similar to bone marrow-derived MSCs; however, there is a difference in marker expression. Bone marrow-derived MSCs are positive for stromal-derived factor-1 (STRO-1), whereas MenSCs are negative for this marker. Because of the many advantages, MenSCs have been rapidly applied in clinical studies. The first report about in vivo application of MenSCs was published by Cui et al. [10]. In this study, the investigators evaluated the effects of both endometrial cells and menstrual blood cells for treating muscular dystrophy in mouse models [10].

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Subsequently, Hida et al. [21] showed that stem cells from both endometrial and menstrual blood can differentiate into cardiomyocyte-like cells. MenSCs significantly restored impaired cardiac function by decreasing the myocardial infarction area in a nude rat model [21]. In mouse stroke models, MenSC transplantation also provided benefits, particularly reduced behavioral and histological impairments [4]. Specifically, these authors showed that the roles of MenSC in stroke treatment did not depend on differentiation of MenSCs into neurons, but the protective effects of growth factors produced by MenSCs [4]. MenSCs have also been used to treat Parkinson's disease. In a 1-methyl 4-phenyl 1,2,3,6-tetrahydro pyridine-induced animal model of Parkinson's disease, it has been shown that labeled MenSCs can engraft, migrate to the lesion site, differentiate in vivo, and significantly increase striatal dopamine and dopamine metabolite concentrations [59]. There is also interest to use MenSCs for ischemia treatment. In a pilot experimental study, [39] showed that MenSC transplantation prevented necrotic ulcers when the femoral artery and its branches were ligated in the hind limbs of rats [39].

Based on these results, we propose that MenSCs can provide many benefits in ischemic disease. MenSCs may stimulate angiogenesis or participate in this process to form new blood vessels and cure this disease. Therefore, our study aims to apply MenSCs to treat hind limb ischemia in mouse models. We hope that MenSCs are a suitable source of stem cells and useful for treating ischemic disease.

MenSC Isolation and Characterization

Menstrual blood was collected using a previously published protocol [46]. Briefly, menstrual blood was obtained from healthy 22–30-year-old women. All donors were required to sign an agreement with our laboratory before donation. To collect menstrual blood, a female volunteer inserted a provided menstrual cup in place of a tampon. This cup was retained for 2–3 h to collect samples because every woman normally gave two to three times of the menstrual fluid. Blood fluid was then carefully transferred to 15-ml Falcon tubes with 2 ml PBS containing a 5× antibiotic-mycotic solution (GeneWorld, HCM, VN). Tubes were kept on ice and quickly transferred to the laboratory. Samples were tested for bacterial and fungal contamination. Only samples negative for both bacteria and fungi were processed further. At the laboratory, menstrual blood was used to isolate mononuclear cells by Ficoll-Paque (Fisher Scientific, Portsmouth, NH), according to the manufacturer's instructions, which were then washed twice in PBS. Mononuclear cells were cultured in T-25 flasks (Nunc, Denmark) containing DMEM/F12 supplemented with 1 % antibiotic-mycotic and 20 % fetal bovine serum (FBS) (Invitrogen-Gibco). Medium was exchanged with fresh medium after 48 h of incubation. At 70–80 % confluence, cells were trypsinized with 0.25 % trypsin/EDTA (GeneWorld) and subcultured in three new flasks. At third passages, cells were used to confirm stem cell characteristics and for further experimentation. After 48 h, some cells adhered to flasks and exhibited a fibroblast-like shape (Fig. 20.1a). Subsequently, cells rapidly proliferated until confluency in 14 days (Fig. 20.1b). These cells were subcultured continuously for ten passages while retaining their morphology (Fig. 20.1c).

Next, specific markers were used to characterize MenSCs, including CD14 (monocyte marker), CD34 (hematopoietic stem cell marker), CD45 (leukocyte marker), SSEA-4 (embryonic stem cell marker), CD9 (MSC marker associated with angiogenesis), CD73 (ecto-5′-nucleotidase, involved in MSC migration), CD44 (hyaluronic acid receptor found on tissue stem cells and MSCs), CD90 (MSC marker), and CD105 (marker of tissue and MSCs) (BD Biosciences, San Diego, CA). Cells were washed twice with PBS containing 1 % BSA (bovine serum albumin) (Sigma-Aldrich, St. Louis, MO). The Fc receptor on the cell surface was blocked by incubation with IgG (Santa Cruz Biotechnology) on ice for 15 min. Cells were stained for 30 min at 4 °C with the antibodies. After washing, cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). The results showed that obtained cells were negative for some markers of hematopoietic cells, such as CD14, CD34, CD45, and SSEA-4 (Fig. 20.2a–d), but

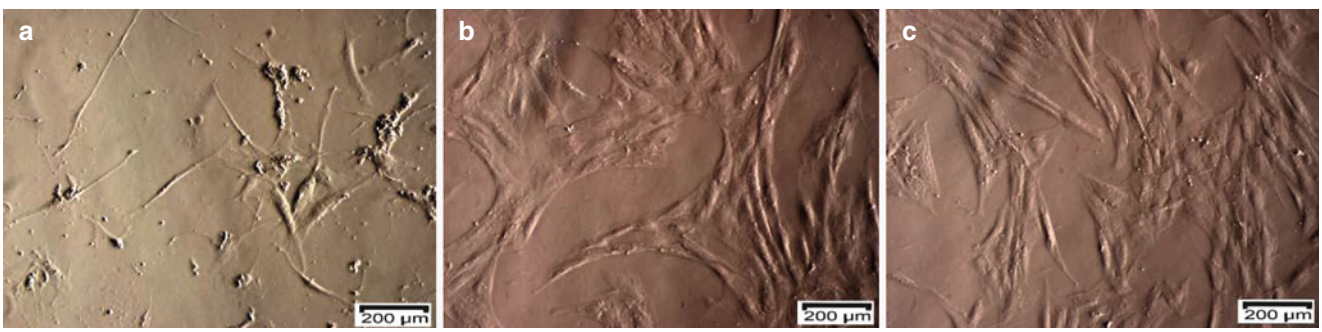

Fig. 20.1 Menstrual blood-derived cells adhered to the flask surface after 48 h of incubation (**a**), rapidly proliferated to confluency (**b**), and maintained their fibroblast-like shape for ten passages (**c**)

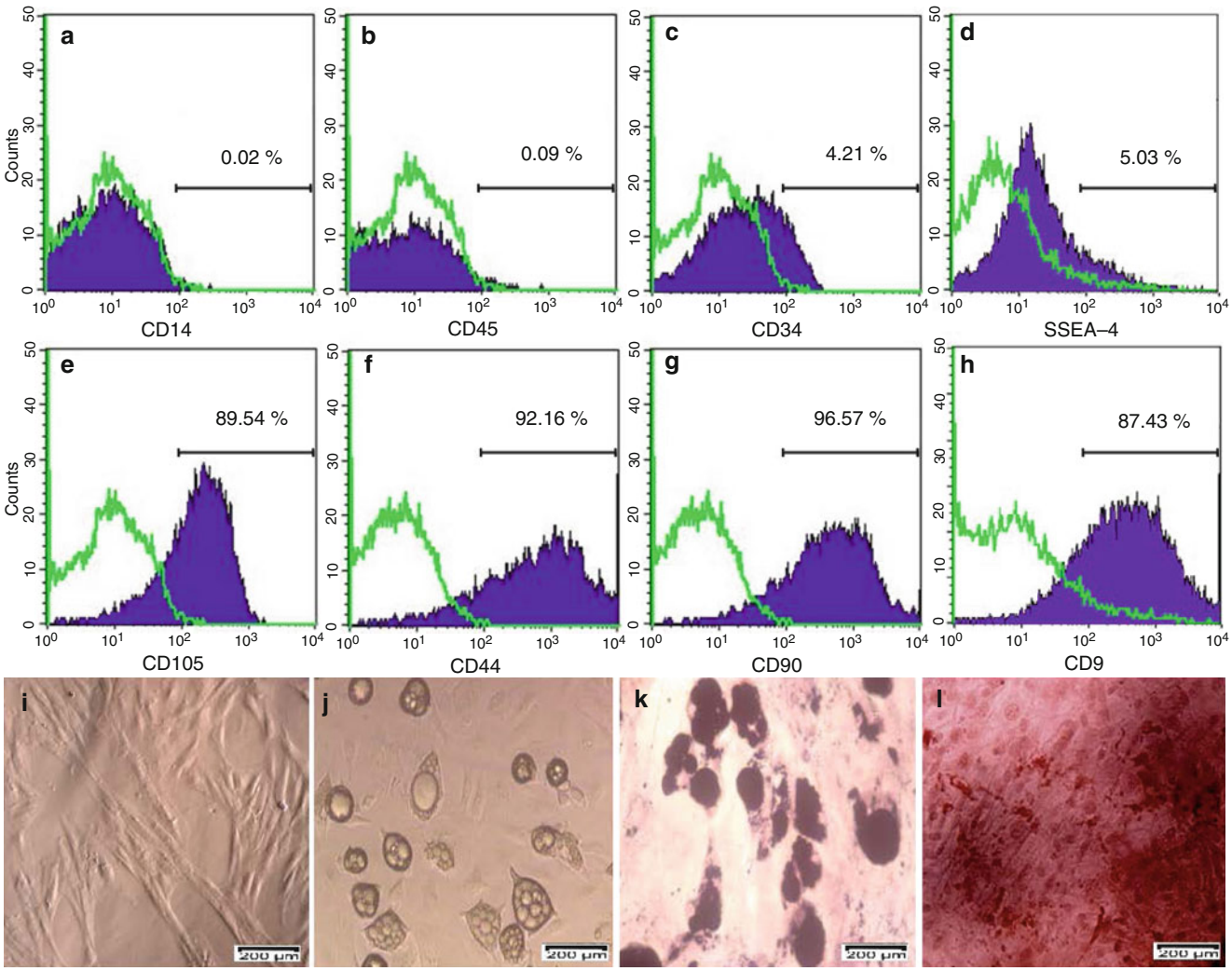

Fig. 20.2 Characteristics of MenSCs. (**a**–**h**) Flow cytometric analysis showed that MenSCs were negative for CD14 (**a**), CD45 (**b**), CD34 (**c**) and SSEA-4 (**d**); positive for CD105 (**e**), CD44 (**f**), CD90 (**g**) and CD9 (**h**). They also exhibited the fibroblast like shape (**i**) and successfully differentiated into adipocytes (**j**–**k**) and osteoblasts (**l**)

positive for typical markers of MSCs, such as CD9, CD44, CD90, and CD105 (Fig. 20.2e–h). Notably, these cells expressed MSC markers associated with angiogenesis. These results demonstrated that MenSCs exhibited an MSC phenotype and the capacity for angiogenesis. In the next experiment, we evaluated the capacity of in vitro differentiation of MenSCs.

For adipogenic differentiation, MenSCs were seeded at a concentration of 5×10^4 cells/ml in a 4-well plate using DMEM/F12 containing 20 % FBS. At confluency, the medium was replaced with adipocyte-inducing medium (GeneWorld), and cells were cultured for 14 days with medium changes every 3 days. After differentiation, cells were stained with Oil Red O (Sigma-Aldrich) and visualized under an inverted microscopy (Carl Zeiss, Germany). For osteogenic differentiation, MenSCs were seeded at a concentration of 5×10^4 cells/ml in a 4-well plate using DMEM/F12 containing 20 % FBS. At confluency, the medium was replaced with osteoblast-inducing medium (GeneWorld), and cells were cultured for 21 days with medium changes every 3 days. Cells were stained with Alizarin Red (Sigma-Aldrich) and visualized under an inverted microscopy. For endothelial differentiation, MenSCs were seeded at a concentration of 5×10^4 cells/ml in a 4-well plate using DMEM/F12 containing 20 % FBS. At confluency, the medium was replaced with osteoblast-inducing medium (GeneWorld), and cells were cultured for 21 days with medium changes every 3 days. Cells were stained with anti-CD34 and anti-CD62 antibodies (BD Biosciences) to determine the differentiation status. The results showed that MenSCs can be differentiated into adipocytes and osteoblasts. MenSCs (Fig. 20.2i) induced with adipogenic medium exhibited the phenotype of adipocytes with numerous lipid droplets in the cytosol after 14 days of induction (Fig. 20.2j). We confirmed lipid droplets by staining with Sudan Black, which became black (Fig. 20.2k). MenSCs were also successfully differentiated into osteoblasts and turned red after stained with Alizarin Red (Fig. 20.2l).

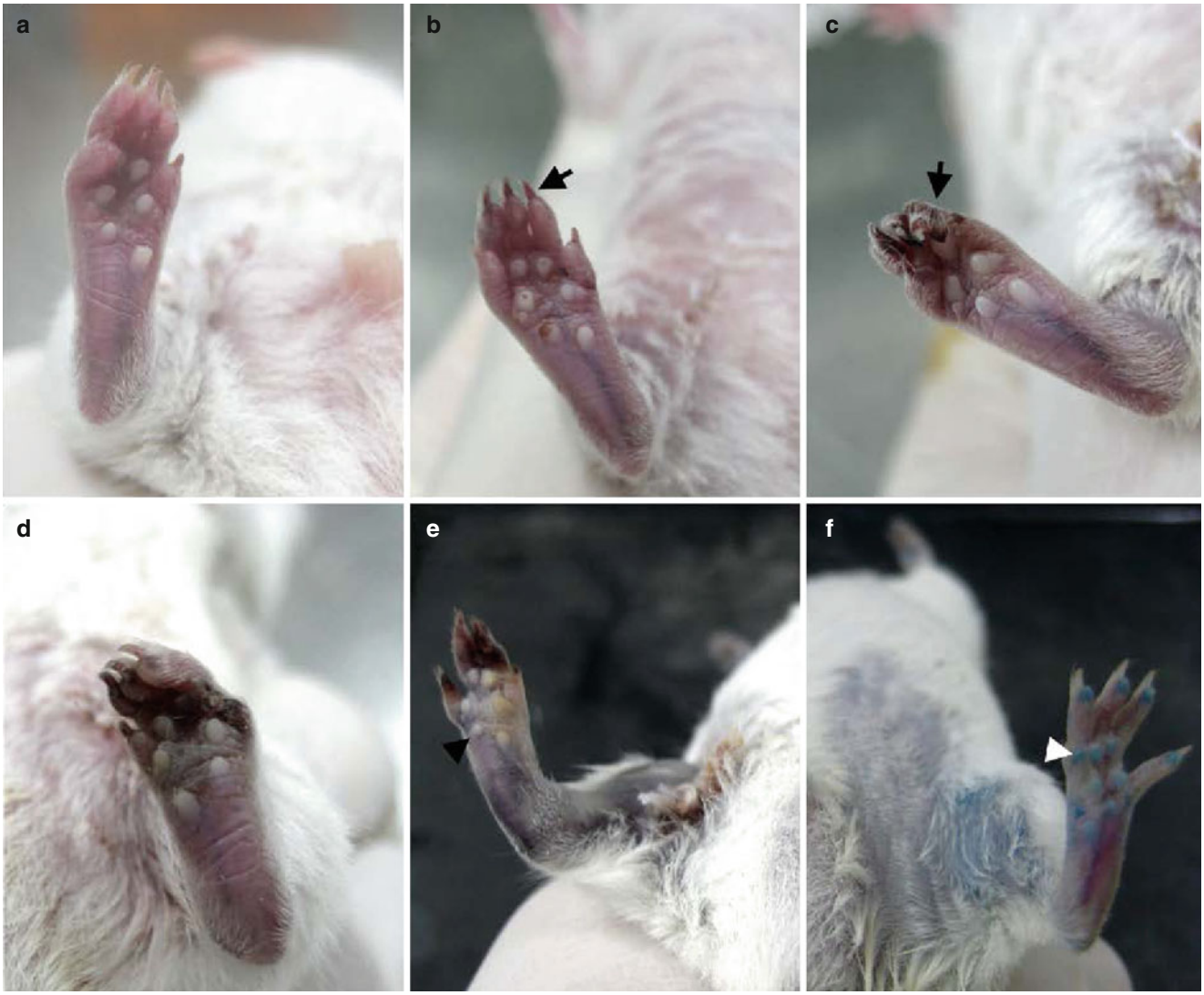

Fig. 20.3 Acute hind limb ischemic mouse models. (**a**–**d**) Morphological changes in the hind limb after the artery and its branches were ligated. After 12–24 h ligation, the hind limb showed cyanosis (**a**), and then the toenails (**b**) and toes (**c**) were necrotized, and the foot became gangrenous (**d**). Mice were checked for blood flow in the artery and its branches by infusion of trypan blue into the portal vein (**e** and **f**). Because blood could not enter the toes after ligation, the toes did not stain blue (**e**), whereas toes became blue in control (**f**)

Hind Limb Ischemic Mouse Models

Hind limb ischemic mouse models were established following the protocol of Niiyama et al. [40]. All procedures on animals were approved by the Animal Welfare Committee of the Laboratory of Stem Cell Research and Application, University of Science, VNU-HCM, VN. Fifty 10–12-week-old mice were used in this experiment to establish ischemic hind limb mouse models. Briefly, mice were anesthetized by ketamine, and then the hair was removed from the hind limb. Using fine forceps and surgical scissors, we made an incision of approximately 1 cm long in the skin. Next, we dissected and separated the femoral artery from the femoral vein and nerve at the proximal location near the groin. After the dissection, a silk suture was passed underneath the proximal end of the femoral artery. The proximal femoral artery was occluded using double knots. A tie was placed on the vessel as proximal to the wound as possible to leave a length for the second tie and an intervening segment that would be transected. The femoral artery was separated from the femoral vein at the distal location close to the knee. A 7-0 suture was passed underneath the distal end of the femoral artery proximal to the popliteal artery. The vessel was then occluded using double knots. Prior to transplantation, all mice were immunosuppressed by cyclosporin A treatment. The efficacy of the protocol was evaluated following the guidelines of Goto et al. [19]. The degree of ischemic damage was recorded and divided into five degrees including 0, I, II, III, and IV, with grade 0, an absence of necrosis; grade I, necrosis limited to the toes; grade II, necrosis extending to the dorsum pedis; grade III, necrosis extending to the crus; and grade IV, necrosis extending to the thigh. Ligation of the femoral artery and its branches resulted in immediate cyanosis in the entire hind limb (Fig. 20.3a). After 24–36 h,

the hind limb began to necrose at the toes, with the toenails and toes becoming black (grade I) (Fig. 20.3b). After another 24 h, the feet became swollen and black (grade II) (Fig. 20.3c). After another 48–72 h, necrosis extended to the knee (grade III) (Fig. 20.3d, e). Finally, after another 96–120 h, necrosis extended into the thigh, and all mice underwent hind limb loss (grade IV).

To confirm that the blood flow could not enter the hind limb, we injected 0.4 % trypan blue into the portal vein. In normal mice (Fig. 20.3f), blood entered the hind limbs, particularly the toes, resulting in the toes becoming blue because of the trypan blue dye, whereas blood could not enter the toes in ischemic hind limb models (Fig. 20.3e). Therefore, we confirmed successfully induced ischemia in mouse models with 100 % efficiency.

MenSC Transplantation and Treatment Evaluation

Acute hind limb ischemic mice were divided into four groups with ten mice in each group. In the first group (G1), mice were used as a negative control (untreated) and injected with PBS. In the second group (G2), mice were injected with a dose of MenSCs into the ischemic region. In the third group (G3), mice were infused with a dose of MenSCs via the tail vein. In the fourth group (G4), mice were injected and infused with doses of MenSCs. A dose of MenSCs contained 5×10^6 cells. All mice were followed up for 4 weeks to evaluate the effects of grafted MenSCs.

The first evaluation was of the degree of ischemic damage. The degree of ischemic damage was evaluated following the guidelines of [19] presented previously. The results showed that, after 4 weeks, there were significant differences between G2, G3, and G4 and G1. In G1, there were 10/10 mice at grade IV (necrosis extending to the thigh), while there were 0/10 mice at grade IV in G2, G3, and G4. Generally, the degree of ischemic damage gradually decreased from G3 to G2 and G4, with the best result obtained with G4. Indeed, grade 0 mice (absence of all ischemic appearance) increased from 0/10 to 2/10 and 3/10 in G3, G2, and G4, respectively. In G4, almost all mice showed reduced ischemic symptoms and increased to grades I and II (Fig. 20.4).

For the second assay, we evaluated the pedal frequency in water over 10 s. Normal and G1–G4 mice were placed

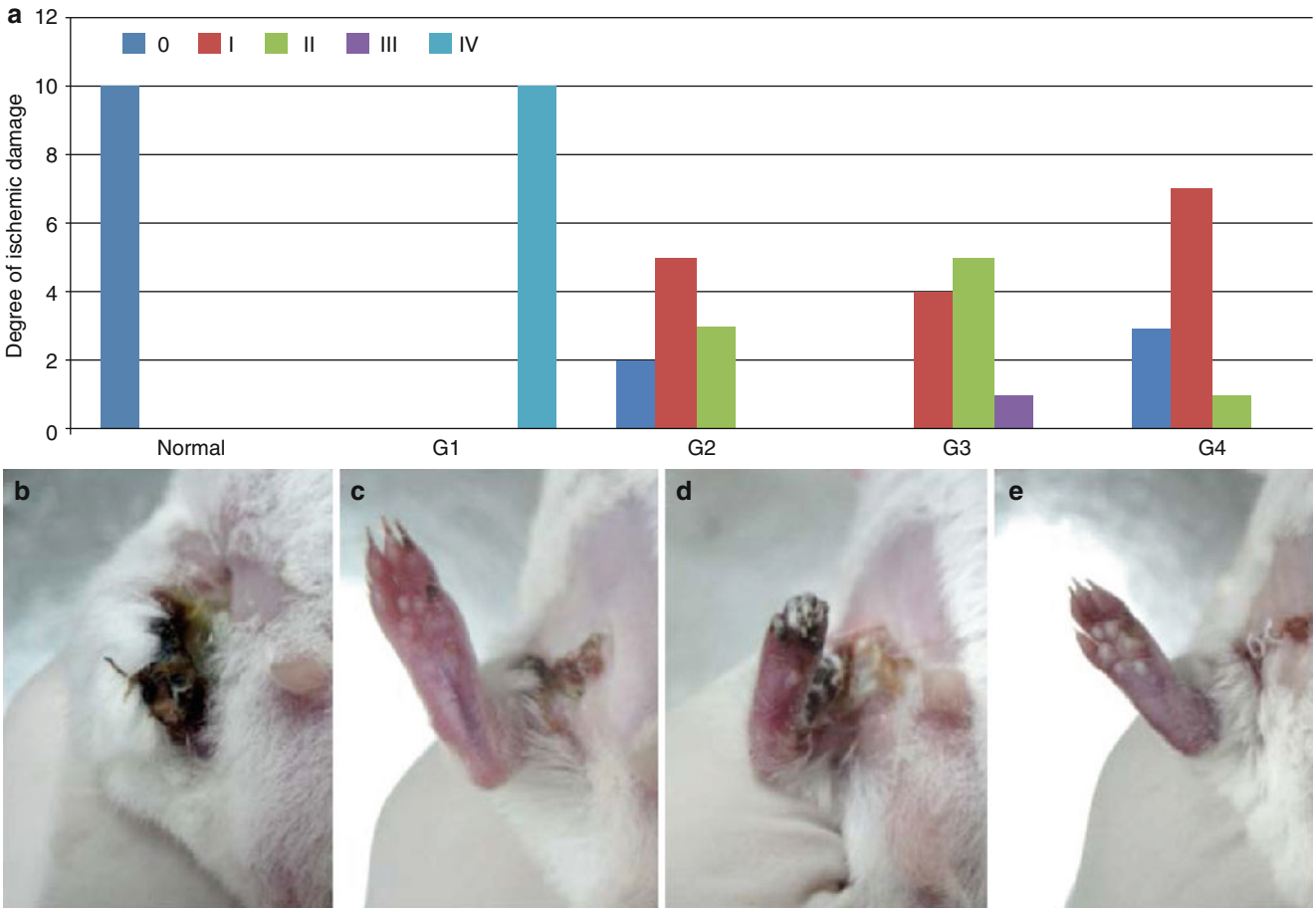

Fig. 20.4 Degree of ischemic damage. The grade of damage was 0–IV and was recorded for the four groups (G1–G4) (**a**). After 4 weeks, all mice in G1 underwent leg loss, while mice in G2, G3, and G4 showed improvement with grades from I to III (**c**–**e**)

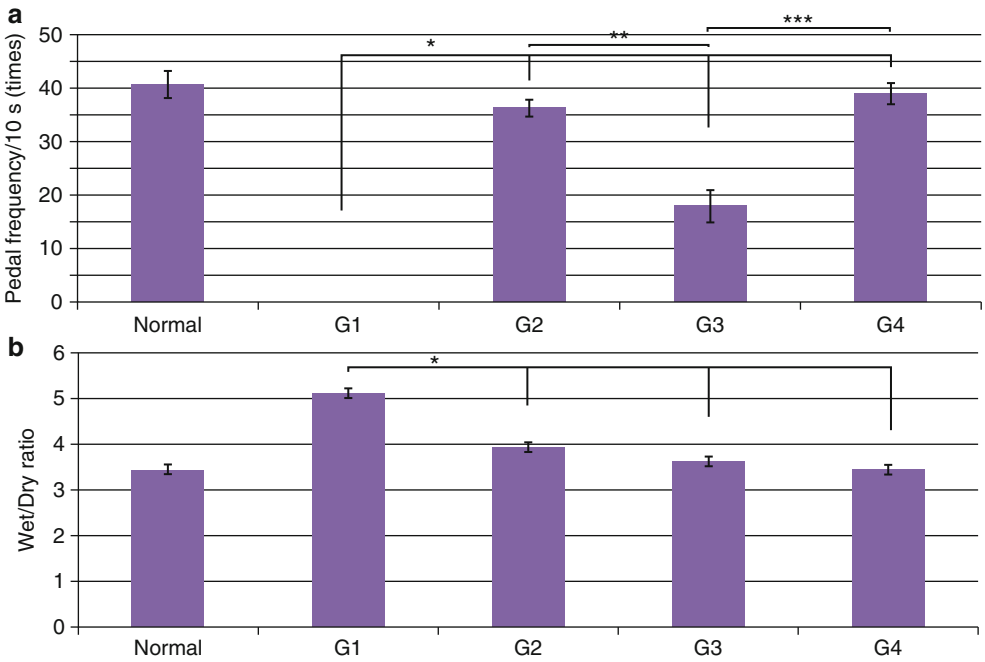

Fig. 20.5 Effects of MenSC transplantation on pedal frequency and the W/D ratio of muscles between experimental groups. (**a**) Pedal frequency increased after MenSC transplantation (G2, G3, G4), compared with that in the control (G1). (**b**) Changes in edema were evaluated by the W/D ratio of muscle. Normal, normal mice; G1, Group 1, untreated ischemic hind limb mice; G2, Group 2, mice were injected with MenSCs into the ischemic region; G3, Group 3, mice were infused with MenSCs via the tail vein; G4, Group 4, mice were treated with both injection and infusion

in water. The natural response was a pedal response in water. We counted the pedal frequency of hind limbs over 10 s. The results are presented in Fig. 20.5a. Normally, the pedal frequency was 40.67 ± 2.52 times/10 s. The pedal frequency in untreated mice was zero, because of their hind limb loss. However, there was a large difference between treated mice (G2, G3, and G4) and untreated mice (G1). MenSC transplantation improved the hind limbs of G2, G3, and G4, which responded when placed in water with pedal frequencies of 36.33 ± 1.53, 18 ± 3, and 39 ± 2 times/10 s, respectively. The pedal frequency in G4 was similar to that in normal mice (40.67 ± 2.52 vs. 39 ± 2), which was higher than that in G2 (36.33 ± 1.53 vs. 39 ± 2) and G3 (18 ± 3 vs. 39 ± 2) ($P > 0.05$ and $P < 0.05$, respectively). These results indicated that MenSC transplantation provided benefits for ischemic hind limbs, improved recovery, regenerated blood vessels, and overcame ischemia. Although there was no statistically significant difference between G2 and G4, MenSC transplantation by intramuscular injection and vein infusion was the best method with complete recovery of hind limbs after 4 weeks. Intramuscular injection was better than vein infusion for same concentration of cells.

The third evaluation was of tissue edema. Tissue edema was evaluated at 1 week after transplantation for G2, G3, G4, and normal mice and at 72 h after ischemia for untreated mice. Mice were examined for evidence of edema after 72 h for G1 and 1 week for G2, G3, and G4. The ischemic muscle (distal thigh and calf muscle) was isolated; tissue samples were weighed and then placed in a drying oven at 55 °C until a constant weight was observed (usually 36–48 h). The degree of muscle edema was quantitated using the wet to dry weight ratio (W/D). The W/D was compared between normal mice and experimental groups. The results showed that tissue edema was strongly decreased in experimental groups (G2, G3, and G4), compared with that in untreated mice (G1). The W/D ratio in normal mice was 3.44 ± 0.11, whereas in untreated mice, this ratio was up to 5.12 ± 0.09. In MenSC transplantation groups, these ratios decreased toward that of normal mice, as shown by W/D ratios of 3.03 ± 0.08, 3.64 ± 0.09, and 3.45 ± 0.08 for G2, G3, and G4, respectively. The differences of W/D ratios between treated and untreated mice were statistically significant, but the differences between G2, G3, and G4 were not significant ($P > 0.05$) (Fig. 20.5b).

The fourth evaluation was of neuromuscular scores. The neuromuscular score was assessed by two blinded observers using a modified clinical score described elsewhere [29]. Neuromuscular scoring was based on motion below the knee (i.e., the calf muscles) and below the ankle (paw-toe motion). There are four grades of neuromuscular scores including 0, full range of motion at the knee (flexion and extension) and paw level (flexion and extension); 1, diminished flexion and extension of the calf, paw extension and flexion are intact; 2, diminished flexion and extension of the knee, paw flexion preserved, no paw extension; and 3, no flexion of the calf, no flexion or extension of the paw (paw drop/dragging). The results showed that the neuromuscular scores decreased gradually from G1, G3, G2 to G4 with 3 ± 0.57, 2.2 ± 0.36, 1.83 ± 0.58, and 1 ± 0.1, respectively. There was a significant difference between G1 and MenSC transplantation groups. Remarkably, the neuromuscular score significantly decreased in G4 to G2 and G3 (Fig. 20.6), indicating that MenSC transplantation reduced ischemic damage, and the combination of intramuscular injection and infusion showed the best results for ischemic regeneration.

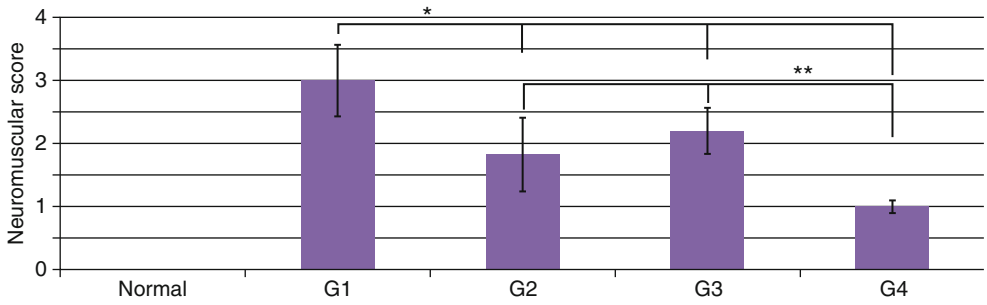

Fig. 20.6 Neuromuscular scores of experimental and normal mice. Neuromuscular scores showed that there were significant differences between G1 and G2, G3, and G4

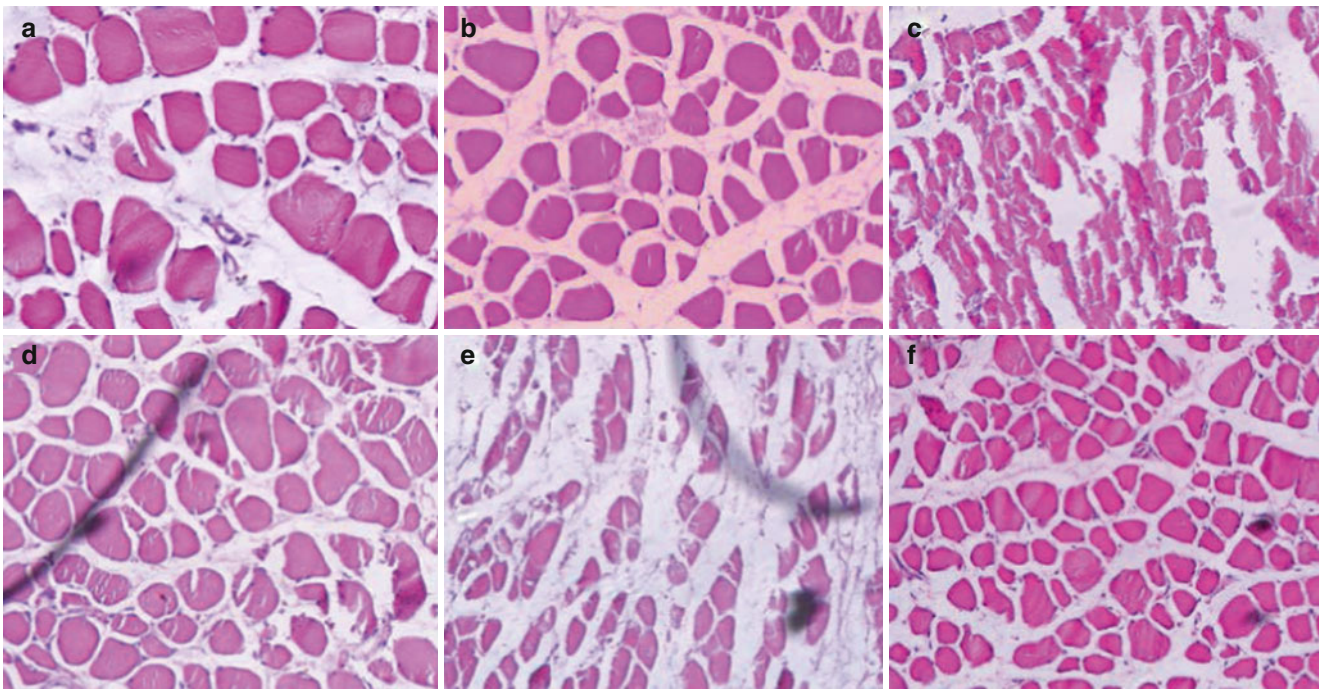

Fig. 20.7 Histology and morphological appearance of ischemic hind limb in experimental groups. (**b**–**c**) Changes in the histology of ischemic muscle analyzed by H-E staining, compared with that in normal mice (**a**). At 24 h after ligation of the artery and its branches, muscle cells became swollen (**b**) and then necrotized after 72 h (**c**). (**d**–**f**) Changes in the histology of muscles in G2, G3, and G4, respectively

The last evaluation was of histology. Limb tissues were histologically analyzed after 4 weeks. Separately, G1 (untreated mice) was evaluated three times, including after 72 and 120 h, because of leg loss after 120 h. Muscles were dissected out, rinsed in PBS for 1 h, and then dehydrated. Samples were cut cross-sectionally at 2 μm thicknesses and then stained with H-E. Stained slides were examined by microscopy at 20× magnification (Carl Zeiss, Germany). Statistical analysis was performed with the means ± standard error of the mean. Comparisons were made using ANOVA and the Student's t-test. A value of $p < 0.05$ was considered significant. Compared with normal mice and untreated mice (G1), we found that there were significant histological differences in muscles stained with H-E. In G1, muscle cells became swollen after 72 h (Fig. 20.7b), compared with normal muscle cells (Fig. 20.7a), and necrotic as indicated by destruction of nuclei, the cytosol, and cell membrane (Fig. 20.7c). In G3, the cell morphology was improved, with several muscle cells exhibiting normal and living morphologies (Fig. 20.7e). The quality of morphologically normal cells increased clearly in G2 and G4 (Fig. 20.7d and f). Moreover, we observed a small number of new myocytes forming in G4, with the cytosol and nuclei stained darker (Fig. 20.7f). These results confirmed the role of MenSC transplantation in ischemic recovery.

Discussion

Ischemia, particularly hind limb ischemia, has become common in recent years. There have been some therapies developed to treat this disease; however, their efficacy is limited. Stem cell therapy offers a new strategy for treating

this disease. Unlike previous therapies, stem cell therapy will regenerate or create new blood vessels to bypass the ischemic area. Therefore, such therapy is considered as the most efficacious for the treatment of ischemia.

In this study, we evaluated MenSC transplantation for recovery of hind limb ischemia in mouse models. In the first experiment, we successfully isolated MenSCs from menstrual blood. Isolated MenSCs showed many characteristics of MSCs, such as expression of typical MSC markers such as CD44, CD90, and CD105. These markers have been confirmed and used to identify and characterize MSCs derived from various sources such as the bone marrow [25, 41, 49, 56], adipose tissue [7, 27], peripheral blood [26], umbilical cord blood [15, 50, 64], banked umbilical cord blood [46], umbilical cord [11, 16], umbilical cord membrane [12, 30], umbilical cord vein [51], Wharton's jelly of the umbilical cord [45, 62], placenta [22, 47, 54], decidua basalis [33], ligamentum flavum [9], amniotic fluid [17], amniotic membrane [8, 35], dental pulp [1, 61], chorionic villi of human placenta [48], fetal membranes [55], and breast milk [44]. With this phenotype, MenSCs are similar to bone marrow-derived MSCs. However, we identified a difference in which bone marrow-derived stem cells express STRO-1 that is used to isolate bone marrow-derived MSCs [20], whereas MenSCs do not express this marker [18]. CD9, a widely expressed membrane protein of the tetraspanin family, has been implicated in diverse functions such as signal transduction and cell adhesion and motility. Moreover, CD9 is essential for angiogenesis, as demonstrated by inhibition of CD9 by an anti-CD9 antibody, which inhibits angiogenesis in metastatic processes [31]. Using adipose stem cells, Kim et al. [28] showed that CD9 has pivotal roles in proliferation and proangiogenic action [28]. CD9 expression by MenSCs indicates the capacity to stimulate neovascularization in ischemia.

In the next experiment, we established hind limb ischemic mouse models. Using the protocol of Niiyama et al. [40], we successfully established grade IV hind limb ischemia in all mice. After 48–72 h, necrosis extended to the knee, and trypan blue infusion showed that there was no blood supply to any blood vessel in the hind limb. Because of leg loss after 96–120 h of artery ligation, in the next experiments, mice were treated with MenSC transplantation immediately.

MenSC transplantation clearly improved ischemia in mouse models. In comparison with untreated mice (injected with PBS), there were significant differences in all physiological characteristics and histology. One of the indicators we were able to easily record to show recovery of ischemia was the degree of ischemic damage. In all MenSC transplantation groups, the degree of ischemic damage was decreased. In almost all cases, ischemic tissue recovered, particularly after MenSC transplantation that combined both injection and infusion, in which some mice were nearly normal. This improvement was confirmed by the pedal capacity in water. After 4 weeks, all mice in G1 lost limbs, whereas limbs were retained in all MenSC transplantation groups. Leg movements in water demonstrated that muscle tissue was alive and functional. In other experiments, we recorded and compared tissue edema and neuromuscular scores of untreated and treated mice. We found an improvement of tissue edema and neuromuscular scores. Mice treated with MenSCs showed significantly decreased tissue edema. This decrease showed that the blood vessels were smooth. Blood can enter ischemic tissue to recover cells. Histological analysis supported these results. All cells in G1 mice were killed by necrosis, whereas MenSC transplantation improved cell survival. In some cases, we found not only living muscle cells but also some new myocytes forming. In addition, neuromuscular transduction was improved. The pedal response in water and neuromuscular scores confirmed this conclusion.

MenSCs may perform some roles in such regeneration. Neovascularization and angiogenesis are the two main processes that recover ischemia. Indeed, after MenSC transplantation, there were many small blood vessels that formed (data not shown). These blood vessels are the result of two processes, namely, vasculogenesis and angiogenesis. Vasculogenesis is the process that forms vascular structures from circulating or tissue-resident endothelial stem cells (angioblasts) that proliferate into de novo endothelial cells. Angiogenesis relates to the formation of thin-walled endothelial-lined structures with a muscular smooth muscle wall and pericytes. These processes play a vital role during adult life as a repair mechanism of damaged tissues. MenSCs can take part in these processes by two mechanisms. The first mechanism is that MenSCs stimulate de novo angiogenesis by secreted factors. Indeed, using MenSC medium, Borlongan et al. [4] showed that MenSCs can produce many factors such as VEGF, BDNF, and neurotrophin-3 [4]. VEGF is one of the crucial regulators of angiogenesis and vasculogenesis. VEGF is an endothelial cell mitogen and angiogenic inducer, and thus it plays an important role in development of the vascular system [24]. In general, stem cells have shown release of some growth factors that stimulate cell proliferation. For example, stem cells obtained from adipose tissue can produce EGF, FGF, and IGF. In the same mechanism, MenSCs have been used to treat neurodegenerative disorders, because they can protect primary neuronal cultures against oxygen/glucose deprivation [4].

The other mechanism relates to the differentiation of MenSCs into endothelial cells that participate in de novo vasculogenesis. MenSCs easily differentiate into endothelial cells when induced in an endothelial induction medium [37]. After transplantation into tissue, MenSCs can differentiate into endothelial cells and trigger vasculogenesis. This mechanism

has not been demonstrated in this study, but Hida et al. [21] showed that MenSCs can differentiate into cardiomyocytes and reduce the size of the myocardial infarct in a nude rat model of myocardial infarction.

In this study, we also showed that intramuscular injection results in better improvement than intra-vein injection. Moreover, the combination of intramuscular injection and infusion into a vein may provide the best outcomes. However, several scores showed no significant differences between intramuscular injection and infusion. These results suggest that MenSCs can directly affect angiogenesis and vasculogenesis. MenSCs in grafted tissue can trigger tissue-specific endothelial cells to proliferate and form new blood vessels. Another reason for the differences in efficacy is the quality of secreted factors that may be lower in ischemic tissue when MenSCs are injected into a vein, rather than direct injection into the ischemic tissue. Moreover, the homing and differentiation of MenSCs when injected into ischemic tissue is more efficient, compared with those of MenSCs injected into a vein.

Based on this study, we propose that MenSCs are suitable for ischemia treatment. MenSCs have many other advantages including safety and a low allogeneic response [2]. Allickson et al. [2] showed that infusion of MenSCs into Harlan Sprague Dawley mice and Dunkin Hartley albino guinea pigs for 7 days showed an absence of extraneous toxic contaminants, and all animals remained healthy, without reactions and showed no weight loss. These authors also showed that MenSCs demonstrate a moderate response in a mixed lymphocyte reaction [2].

Conclusion

Menstrual blood is a rich source of stem cells. The stem cells derived from this source of blood exhibit many typical markers of MSCs. Moreover, they express the angiogenesis marker CD9. Transplantation of MenSCs clearly reduces ischemia in hind limb ischemic mouse models. MenSCs assist angiogenesis and vasculogenesis in which many new blood vessels are formed to supply nutrients to muscle cells. Such recovery involves not only survival of muscle cells but also their physiological activities, particularly movements and neuromuscular transduction. Taken together with other advantages, MenSCs are suitable candidates for treating ischemia in both autogenic and allogeneic transplantation.

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Part VI

Clinical Applications

Of Baul Behavior, Stem Cell Primitive Application?

21

Niranjan Bhattacharya, Sushanta Kumar Banerjee, Sanjukta Bhattacharya, and Phillip G. Stubblefield

Bauls are a secretive sect in rural Bengal who live in their own social groups/communes and follow their own norms. The Bauls of Bengal have their own syncretic religious beliefs largely based on ideas taken from Hinduism, Islam, and Buddhism. Bauls disregard social constraints such as the caste system, and this allows them freedom to achieve liberation through their own versions/perceptions of the realization of the Divinity [1–3].

Lalan Fakir, who is one of the oldest recorded writers of Baul songs, gives an indication of the beliefs and practices of the Baul cult through his songs, and this is a major source of their ideas since there is no written record of their beliefs. In the Baul religion, individual inquiry and emphasis on the importance of a person's physical body are important because Bauls believe that it is within the body that the Supreme resides and thus it is the only place people need to search for God [4, 5].

The word "Baul" comes from the Sanskrit root *vatul*. It means mad, affected by wind. The Baul belongs to no religion. He is a simple human being. He is a social rebel and his rebellion is total. He does not belong to anybody; he lives in a no man's land: no country is his, no religion is his, no scripture is his. His rebellion goes even deeper than the rebellion of the Zen Masters – because at least formally, they worship the Buddha. Formally, they have scriptures – scriptures denouncing scriptures – but scriptures all the same.

Bauls, on the other hand, have nothing – no scriptures, no church, no temple, no mosque. A Baul is a man always on the road. He has no house, no abode. God is his only abode, and the whole sky is his shelter. He possesses nothing except a poor man's quilt, a small, handmade one-stringed instrument called *aektara*, and a small kettle-drum. He possesses only a musical instrument and a drum, which he plays with one hand on the instrument while beating the drum with the other. A peculiar dance with special steps is also part of his way of life on his way to liberation [4].

The Baul does not even use the word "God." The Baul word for God is *adhar manush*, the essential man. To find that *adhar manush*, that essential man, is his search. There is no need for places of worship because according to them, God is inside you and your body is the temple [5].

The Baul's religion is therefore an individualistic approach towards truth. His is an inclusive world where nothing is taboo including sex, *Samadhi*, and certain practices which would be considered shocking in the ordinary world. According to Bauls, "Everything comes from that essential core of your being, so why deny it? And if you deny it, how will you be able to reach to the source?" To the Baul, therefore, denial means obstruction in the path to salvation [6–8].

This does not imply that a Baul is a man of mere indulgence. They have secretive practices, and only those who are inducted into the sect get to know the rituals which, in fact, led to an earlier ostracization of the sect. From the little that is known of these rituals, some strange facts emerge that correlate to some of the emerging understandings of stem cell science. It is widely known that Bauls rarely have children, and they enjoy long periods of healthy, active life. They appear to know the path to transforming something that is base and mundane into something higher, that is, that menstrual blood and lactating milk contain something that helps

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in keeping the body youthful. Their very act of ritualistic sex, though symbolic, may have implications that have their roots in science.

The Baul songs speak of "four moons"; these are used in ritual sex and apparently are stool, urine, menstrual blood, and semen. These respectively represent the four gross elements pervading the world of senses: earth, water, fire, and air. These "four moons" are usually gathered in a ritual pot and mixed with cow milk, coconut milk, camphor, and sugar and consumed by both male and female practitioners before the ritual sex, which is practiced in such a way as to control every physical impulse in order to experience feelings as "natural" or "coemergent" and to be freed from egotism. Bauls believe that the *moner manush* (literally, the man of the mind, or God in a symbolic sense, because He, after all, dwells in the person) resides in the "lotus-shaped flower" at the top of the head (which coincides with the cortex in physiological terms). Sexual activity makes it (the *moner manush*) swim down in the form of a fish, on to the perineum, where it is empowered by the female energy. Once the feminine element has awakened the masculine one, the fish will move back towards the head piercing all the chakras (which are different circles of different shapes and sizes according to Hindu *yogic* beliefs, situated at different vital spots of the spine and body and correlate to nerve centers; it is necessary according to *yogic* beliefs to raise them in order to gain realization of the Self). This ritual sexual intercourse is supposed to take place on the third day of the female companion's menstruation, which is symbolized as the day of the new moon [9–11].

According to the Baul sects' understanding, three streams of blood that constitute a woman's menses (i.e., the water of compassion, the young water, and the charming water) join together in the female genitalia to form a triveni (trident or a meeting of three rivers). Swallowing menstrual blood is a part of a secretive Baul ritual. Further, the oldest Baul in a group enjoys the youngest postnatal lactating Baul mother's milk after the newborn has had its share [12]. Practicing Bauls, interestingly, are never fat and look younger than their age, and are lean, thin, and agile in their physical appearance.

Although it is too early to come to any scientific conclusion or even to speculate, some facts about menstrual blood and mother's milk that are no secret to the scientific world today may be noted. Stem cells are present in menstrual blood as well as in human milk, and their effective use is well documented in many scientific studies. Whether the Bauls, who are simple semiliterate and even illiterate people who originated 500–600 years before these scientific facts about menstrual blood or mother's milk began to emerge, knew about the potential properties of these materials cannot be proved in the absence of any literature and the secretive and obscurantist nature of the sect, but they certainly seemed to have stumbled onto something which they incorporated in their rather iconoclastic rituals, giving it symbolic meanings which are difficult for the layman to understand. One may try to rationalize the menstrual blood swallowing as an aberrant sex behavior or pheromone-mediated (?) behavior specific to the Baul cult. This behavior is also seen in mammals and reptiles. In such cases, pheromones may be detected by the vomeronasal organ (VNO), or Jacobson's organ, which lies between the nose and mouth and is the first stage of the accessory olfactory system. Some pheromones in these animals are detected by regular olfactory membranes.

In the human system too, there are three axillary steroids that have been described as human pheromones: androstenone, androstenol, and androstadienone. Women tend to react positively after androstadienone presentation, while men are more negative [13]. The axillary steroids are produced by the testes, ovaries, apocrine glands, and adrenal glands. In the female species, a class of aliphatic acids was found in female rhesus monkeys that produced six types in the vaginal fluids. The combination of these acids is referred to as "copulins." One of the acids, acetic acid, was found in all of the sampled female's vaginal fluid. Even in humans, one-third has all six types of copulins, which increase in quantity prior to ovulation [14].

As yet, the science behind these practices is not known, but as mentioned, Bauls have been observed to look younger and act more agile in relation to their age. More research may prove the relation between their ritualistic practices and their apparent youth. Whether stem cells have anything to do with the Baul phenomenon can be a subject of further study.

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Application of Umbilical Cord and Cord Blood as Alternative Modes for Liver Therapy

22

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Summary

Regenerative medicine has recently shown promise in the management of various human diseases. Recent reports of stem cell plasticity and multipotentiality have raised hopes of stem cell therapy offering exciting therapeutic possibilities for patients with chronic liver disease. With the understanding that stem cells might not just be about making organs ex vivo, but also regenerating a patient's own tissues, a concept is now developing to use stem cells to treat patients with serious disease conditions that are terminal or where conventional modes of treatment are insufficient.

Liver cirrhosis and/or liver malignancies have been nominated as the 5th leading cause of death worldwide. The WHO reported, in 2006, that 20 million people around the globe suffer from some form or other of severe liver illness. The ultimate fate of end-stage liver disorders is hepatic dysfunction and eventually organ failure. The only curative mode of management for liver failure is liver transplantation, which is subject to many limitations. Novel alternatives, such as artificial and bioartificial support devices, only aid in temporary replacement of some liver function until an organ is available for transplantation. These newer modalities also have drawbacks or remain experimental and still demand further controlled trials to allow proof of concept and safety before transferring them to the bedside. There exists a choice of stem cells that have been reported to be capable of self-renewal and differentiation to hepatobiliary cell lineages both in vitro and in vivo. It may, however, be argued that with a global population of six billion people and a global birth rate in access of 130 million per year, the products of birth, umbilical cord and cord blood possibly provide the most readily accessible and ethically sound alternative source of stem cells. The differentiated stem cells can be potentially exploited for gene therapy, cellular transplant, bioartificial liver-assisted devices and drug toxicology testing and use as an in vitro model to understand the developmental biology of the liver.

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The Afterbirth as a Stem/Progenitor Cell Resource: A Summary

Foetal stem cells comprise a broad stem cell class and can be isolated from two distinct sources, the foetus proper and the extraembryonic structures. The extraembryonic tissues are a rich stem cell reservoir with many advantages over both embryonic and adult stem cell sources. The afterbirth is routinely discarded at parturition, thus allowing little ethical controversy over harvesting of resident stem cell populations. In addition, the extracorporeal nature of these stem cell sources assists isolation, eliminating patient risk that may accompany adult stem cell isolation. Furthermore, the relatively large volume of extraembryonic tissues and the ease of physical manipulation theoretically interpret as an increase in the number of stem cells isolated.

Umbilical Cord Blood

The first isolated foetal stem cells were haematopoietic, derived from human umbilical cord blood. Thus, cord blood represents the prototypic foetal stem cell source. UCB-derived stem cells enjoy an intermediate niche between ES and ADS with the added advantages of being ethically sound and associated with completely non-invasive methods for collection of the cord blood. Until the advent of cord

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blood banking in the mid-1990s, the placenta and umbilical cord were considered clinical by-products, but ever since cord blood has created hope as a new alternative in the management of various disease conditions for which there is no cure or the current treatment options are inefficient. Cord blood provides an easily available and rich source of haemopoietic and non-haemopoietic stem cells. Term and preterm umbilical cord blood contains an equal or significantly higher number of early and committed progenitor cells when compared with bone marrow and greatly surpasses that of adult peripheral blood [5]. Much work has been done in the area of exploring potential of stem cells in cord blood. One such exciting study is the work done by McGuckin et al., who investigated the multipotential capability of cord blood-derived stem cells [6, 7]. In 2005, McGuckin et al. reported the world's first reproducible production of cord blood-derived stem cells expressing embryonic stem cell markers [75]. Their work was supported by other reports concerning the existence of circulating embryonic stem cell-like cells during foetal development [190, 191].

Another population of non-haematopoietic multipotent stem cells termed mesenchymal stem cells (MSCs) has been identified in UCB [8–12]. These multipotent cells [13] have unique immunoregulatory features that suppress lymphocyte proliferation in vitro [14] and exhibit high self-renewal potential [15]. MSCs have potential to differentiate into tissues of all three germ layers: including bone, cartilage, fat, muscle, endothelial cells, neuronal, glial cells and liver [8, 16–35].

Although full-term UCB is reported to be a source of MSCs [8–12], this source of MSC is subjected to a lot of controversy [9, 10, 16, 35–39]. Kogler et al. reported that the amount of MSC in UCB was one MSC in every 20 ml of UCB [10]. Additionally, previous studies have demonstrated that only in 63 % of UCB units could MSCs be isolated in culture with a frequency of MSC-like cells ranging from 0 to 2.3 clones per 1×10^8 MNC cells [11]. This makes the isolation of UCB-sourced MSCs very much dependent on the quality and volume of the cord blood, hence making cord blood not a very efficient resource for MSC compared to the umbilical cord.

Umbilical Cord

The umbilical cord (UC) has recently been recognised as a rich source of stem/progenitor cells, namely, mesenchymal stem cells [40–42] (Fig. 22.1). Wharton's jelly, first described by Thomas Wharton in 1656 [43], is the matrix filling the umbilical cord and is a rich source of MSCs. Chacko and Reynolds [44] described these cells residing in Wharton's jelly as smooth muscles, but Takechi et al. [45] refined the description to 'myofibroblasts' after in situ labelling of vimentin, desmin, α-actin and myosin, which was confirmed by Kadner et al. [46]. These cells are present in relatively high numbers, with an average of 400,000 cells isolated per umbilical cord [47]. This is significantly greater than the yield from adult bone marrow. These cells grow adherent to plastic and express specific patterns of cell surface determinants (CD105, CD90, CD73 and CD44). This cell fraction is distinct from the haematopoietic and pluripotent stem cells present in cord blood and does not express blood-cell determinants (CD34, CD45). These mesenchymal cells can be expanded very efficiently in vitro and have been propagated in culture for more than 80 population doublings [48].

In vivo studies involving the transplantation of UC MSCs are limited, but encouraging. These cells have been transplanted into animal models of disease (severe muscle damage, ischemic rodent brain and hemiparkinsonian) and have demonstrated very promising results [49–51].

Amniotic Fluid

Of all the extraembryonic sources, amniotic fluid is the only one where harvest occurs prior to parturition. This trait may prove increasingly important as in utero cell-based therapies progress [52]. Amniotic fluid contains a heterogeneous population of cells displaying a range of morphologies. Most of these cells are epithelial in nature and have a limited capacity to proliferate in nature. These cells are predominantly derived from the foetal skin and urogenital, respiratory and digestive tract. Additional cells derived from the inner surface of the amniotic membrane add to the mix. The cellular composition of amniotic fluid changes with gestation, coinciding with the maturation of the foetus [53]. Stem cells with the characteristics of HSCs within amniotic fluid were first isolated and described in 1993 by Toricelli et al. [53]. However, non-haematopoietic mesenchymal stem cell population within amniotic fluid was only recently reported [54]. A subset of these cells have reportedly tested positive for Oct4 expression highlighting their possible pluripotent nature [55]. It has been reported that these MSCs express both mesodermal and ectodermal gene products. This characteristic is consistent with the emerging concept that stem cell populations exist in a multi-differentiated state. The proliferation capacity of these cells meets or exceeds that described for adult human MSCs. Additionally, these cells have demonstrated the ability to differentiate into various tissues derived from all three germ layers [54, 56–58]. Amniotic fluid cells have been transplanted into animal models of disease but have produced contradictory results [59, 60]. More work is demanded to resolve these apparently contradictory outcomes and to pronounce amniotic fluid MSCs as a possible alternative for cell transplantation therapies.

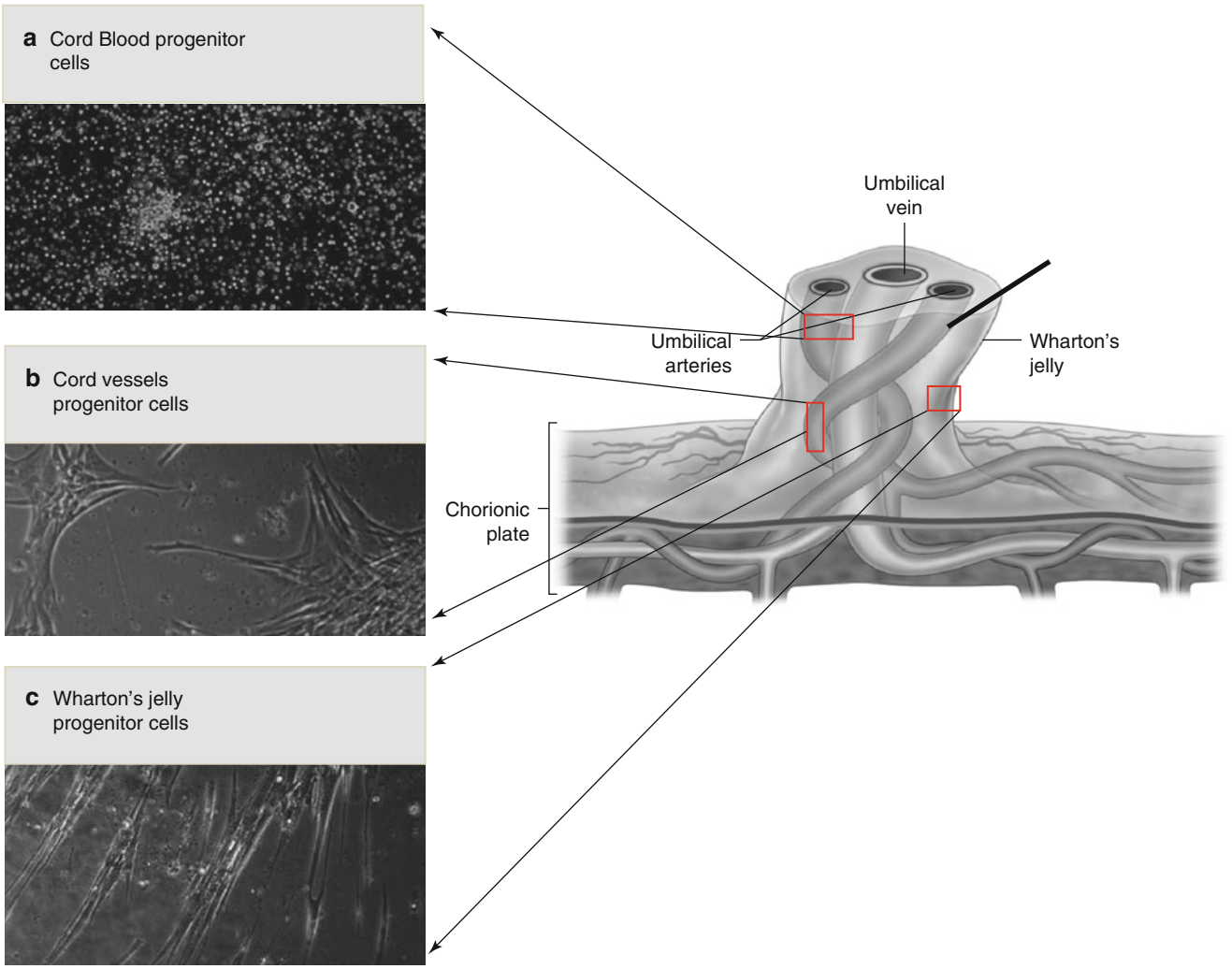

Fig. 22.1 Stem/progenitor cells derived from the umbilical cord. (**a**) Populations of cord blood cells, these cells have a tendency to form aggregates upon culture in vitro. (**b**) Cord vessel progenitor cells with starlike phenotype. This phenotype is lost after the first passage subsequent to which the cells take on a standard MSC phenotype as shown in (**c**)

Placenta

This large fetomaternal organ is an attractive tissue for the isolation of stem/progenitor cells. The sheer volume of a full-term placenta, weighing more than 500 g, almost 15 times the weight of the average umbilical cord makes it a rich source of multipotent cells. MSCs have been isolated from dissociated placental tissue based on plastic adherence. These cells express the pluripotency markers Oct4 and Rex1. These cells exist in a multi-differentiated state – simultaneously expressing ectodermal, mesodermal and endodermal genes. These cells have demonstrated tremendous ex vivo proliferation capacity and the ability to differentiate into tissue representing all three germ layers [32, 61, 62]. Placental-derived MSCs have also demonstrated the ability to support the growth of exogenous HSCs derived from cord blood in culture [63]. Placental-derived MSCS have been utilised in ex vivo engineering of complex biological structures such as artificial heart valves [64]. Additionally, these cells have also been exploited in in vivo studies on animal models and demonstrated interesting results [10].

Amniotic Membrane

The amniotic membrane or the amnion is one of the first recognisable tissues derived from the epiblast. This tissue is an avascular structure generated early in development from multipotent cells residing in two distinct areas of the developing blastocyst. During subsequent development, the cells of the amnion are not exposed to the same signals responsible for the gradual fate restriction of cells within the embryo proper. It is thus postulated that the stem cell populations sequestered within the amnion might retain the potency of the epiblast cells from which they are derived.

Several multipotent cells have been isolated from the amnion, including MSCs [48] (Fig. 22.2). Amniotic membrane has been extensively used as a biological dressing to treat chemical and thermal burns. The clinical success of amniotic membrane transplantation is greatly due to its immunoprivileged characteristics. If this trait is indeed shared by its constituent stem cell populations, they may prove very useful for allogeneic transplantation strategies [65, 66]. In addition, these cells secrete a number of immunosuppressive factors that target the innate and adaptive immune systems, which may support long-term survival of grafts following transplantation [67, 68]. Some of the isolated stem cell populations were reported to be positive for pluripotency markers and demonstrated the capacity to differentiate into tissue derived from all three germ layers [69]. Amnion-derived stem/progenitor cells have been transplanted in animal models of brain diseases and have illustrated promising results [70–72].

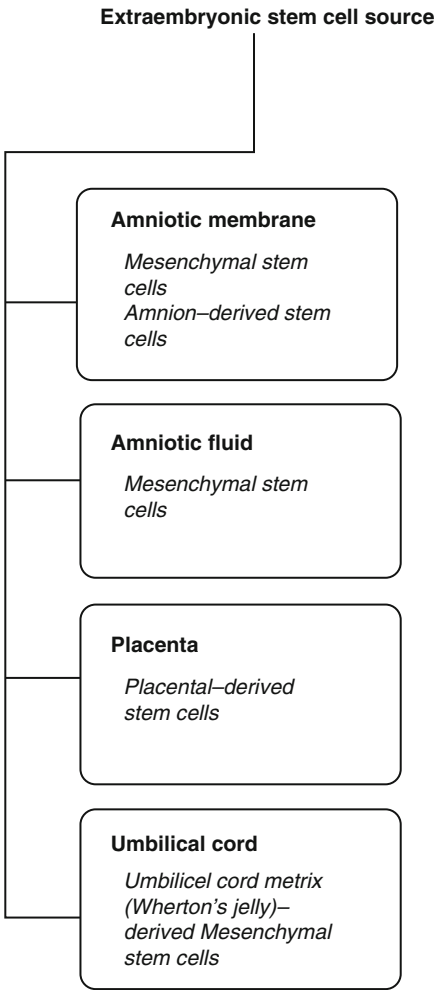

Fig. 22.2 Extraembryonic stem cell sources. The diagram illustrates that stem/progenitor cells have been isolated from all extraembryonic tissues including: the amniotic membrane, amniotic fluid, placenta, and umbilical cord

Umbilical Vessels

MSCs have also been identified in umbilical vessels [73, 74].

Stem/progenitor cells derived from cord blood have been extensively studied and have demonstrated clinical utility for more than 20 years. More recently the umbilical cord has gained a lot of attention over other extraembryonic tissues as an alternative stem cell source for cell transplantation approaches.

It may be safely concluded that cord- and cord blood-derived stem/progenitor cells offer multiple advantages over truly adult stem cells and over embryonic stem cells. With a global population of six billion people and a global birth rate in access of 130 million per year, umbilical cord and cord blood possibly provide the most readily accessible and ethically sound alternative source of stem cells [75]. Although UCB contains fewer primitive haemopoietic precursor cells than earlier trimester foetal bone marrow, it still provides a source of stem cells at a younger stage than mobilised peripheral blood (MPB) and bone marrow [76].

Other differences between these stem cell sources include UCB, and MPB (mobilised peripheral blood) haemopoietic precursors are slowly cycling, whereas those in foetal and adult bone marrow have a higher proportion in G_2, M and S phases. In terms of ontogeny, UCB cells are at the intermediate point between embryonic and adult life and have longer telomeres and high proliferation potential. In addition, UCB has an increased capacity for self-renewal [77]. UCB units also have a reduced risk of viral contaminations (cytomegalovirus or Epstein-Barr virus) [78–80].

Haematological transplantation using UCB further demonstrated a lower incidence of graft-versus-host disease for allogeneic graft [81, 82]. UCB induces better tolerance for human leukocyte antigen (HLA) mismatches when compared to bone marrow, probably due to the immaturity of the immune cells or controlled by UCB dendritic cells and/or natural killer cells [83, 84]. Less stringent criteria for HLA donor-recipient selection, tolerance of 1–2 HLA mismatches out of 6. This allows extension of the donor pool. They may be the only source of allogeneic HSCs (haematopoietic stem cells) available to patients with rare HLA types and hence to ethnic minorities, to siblings suffering from diagnosed haematological disorders and for urgent unrelated donor transplants. Logistical and clinical advantage: prompt availability of transplant; patients receive UCB transplants in a median of 25–36 days earlier than those receiving BM [85].

Clinical Application of Cord Blood

The past 30–40 years have seen great successes in HSC therapies. These provide curative therapies for patients with haematological and non-haematological disorders. Currently more than 85 diseases are either treatable with or

ameliorated with the use of umbilical cord blood. The present day is witness to more than 4,051 stem cell-based therapies in early human trials around the world, based on the Clinical Trials Database (www.clinicaltrials.gov). The beginning of stem cell research dates back to 1866, when Ernst Neumann first postulated the bone marrow as a blood forming organ with a common stem cell for all haematopoietic cells. Further studies followed to increase the understanding of early mammalian development, and more recently substantial work has been done in the area of stem cell biomedical research to help improve our understanding of how stem cells influence the organism to maintain its homeostasis.

Twenty years ago few diseases were treatable with cord blood, 10 years ago about 10–15 and now over 85 diseases. However, the history of cord blood (Fig. 22.3) is long with the first clinical use reported in the 1930s, where it was suggested for use as a substitute for peripheral blood for performing transfusions [2]. HLA matching was not available then and no adverse effects were noted; hence, feasibility of cord blood administration to a non-preconditioned host was suggested. Later, the first traceable umbilical cord blood transplant was performed in 1970 in a 16-year-boy with acute lymphoblastic leukaemia [1]. This procedure is less cited in literature. The boy received cord blood units from eight different unrelated donors, largely untested for true HLA compatibility. Only one unit engrafted, but the patient remained in remission with maintenance chemotherapy until his last follow-up appointment at 9 months. The results of this transplant fuelled research into umbilical cord blood as a stem cell resource and in the 1980s it was reported that umbilical cord blood contained haematopoietic stem cells that may be suitable for transplantation.

In 1988, the first reported cord blood transplantation was executed to cure Fanconi's anaemia in a 5-year-old boy, using blood from his HLA-identical baby sister's umbilical cord, in Paris, France [4]. This report leads to the establishment of cord blood banks worldwide for the collection and cryopreservation of cord blood for allogeneic haematopoietic stem cell transplants.

Since that first reported cord blood success 20 years ago, the number of umbilical cord blood transplantations (UCBT) from siblings and unrelated donors has increased dramatically. In 2006, it was estimated that more than 5,000 patients had undergone UCBT from unrelated donors [86]. By 2009, 20,000 UCBT were performed in children and in adults [3].

In 2006, the inventory of Netcord, the cooperative network of large experienced UCB banks, had over 100,000 cryopreserved UCB units ready for clinical use, and a reasonably accurate worldwide estimate would be 250,000 UCB units [86]. By 2009 that number had increased to 400,000 in more than 50 banks worldwide (www.bmdw.org) [3]. A survey of the International Bone Marrow Transplant Registry (CIBMTR) estimates that after 1998, 20 % of stem cell transplants performed in young patients (<20 years) were cord blood transplants. In Japan, approximately 50 % of haematopoietic stem cell transplants from unrelated donors were performed with cord blood cells. This development was aided by organisation of international registries for outcome

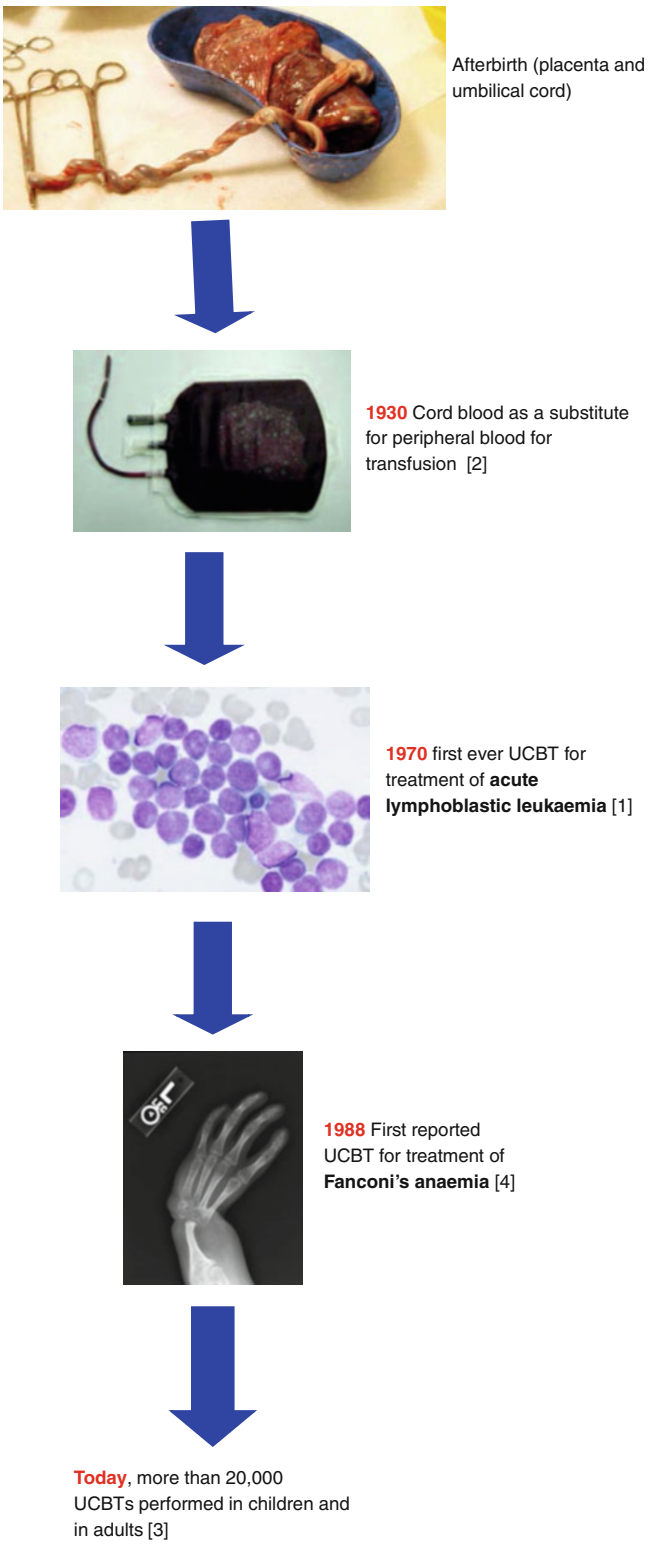

Fig. 22.3 History of umbilical cord blood clinical applications

data collection, including Eurocord (www.eurocord.org) and CIBMTR (www.cibmtr.org) and of cord blood bank networks including Netcord (www.netcord.org) and NMDP (www.nmdp.org) [3].

Umbilical Cord Blood Transplantation Criteria and Consideration for Liver Disease

Umbilical cord blood transplantation criteria and consideration for liver disease: three factors are key determinants for allogeneic engraftment in haematotherapy: number of nucleated cells (TNC) and $CD34^+$ cells infused and the number of HLA differences [87, 88]. The latest recommendations are to choose UCB units with ≤2 HLA disparities and >2.5 × 10^7 NC/kg or ≥2 × 10^5 $CD34^+$cells/kg. In non-malignant diseases where the risk of rejection is higher, the dose should be increased and units with <3.5 × 10^7 NC/kg and two or more HLA incompatibility avoided. If a single UCB unit proves insufficient, then two units with a combined total dose of ≥3 × 10^7 TNC/kg and if possible not more than 1 HLA difference between the two units and the patient should be used [3].

Cord blood application was initially limited to treatment of paediatric haematological malignancies due to low cell yield from cord blood units of modest volume. Two discoveries aided the advance of cord blood beyond paediatric haematology: (i) the need for less strict HLA donor-recipient selection permitted cord blood to be applied in adult patients lacking BM donors [89–92] and (ii) the discovery of early non-haemopoietic stem cells [6, 7]. Outside the area of oncology, clinical use of cord blood expanded into various areas of disease management: reconstitution of a defective immune system, correction of congenital haematological abnormalities and inducing angiogenesis. Table 22.1 illustrates some

Table 22.1 Non-malignant disorders treated with cord blood

Disorder	Number treated	Outcome	Reference
Inborn error of metabolism			
Hurler's disease	20	17 out of 20 children alive a median of 905 days after transplantation with complete donor chimerism and normal peripheral blood α-L-iduronidase activity	[94]
Mucopolysaccharidosis type IIB (Hunter's syndrome)	1	2 years post UCBT ~55 % normal plasma iduronidase sulfatase activity was restored and abnormal urinary excretion of glycosaminoglycans was nearly completed resolved	[107]
Chronic granulomatous disease			[109]
Immune deficiency disorders			
Wiskott-Aldrich syndrome			[108]
Muscular dystrophies			
Duchenne muscular dystrophy	1	On day 42 post transplantation, obvious improvement in walking, turning the body over and standing up was noted	[95, 96]
Bone disorders			
Malignant infantile osteoporosis	1	Normalisation of spine bone mineral density was noted	[97]
Vascular disorders			
Buerger's disease	4	Ischemic rest pain suddenly disappeared. Digital capillaries were increased in number and size	[98]
Degenerative disorders of the nervous system			
Spinal cord injury	1	Within 41 days post transplantation, improvement in sensory perception and movement in the SPI patient's hips and thighs was noted. Regeneration of the spinal cord at the injured site	[99]
Krabbe's disease	25	Progressive central myelination and continued gains in developmental skills, and most had age-appropriate cognitive function and receptive language skills in patient subset	[100]
Omenn syndrome	1	T-cell reconstitution	[101]
Non-healing wounds	2	Accelerated healing	[102]
Blood disorders			
Refractory anaemia	3	All patients were alive and free of disease at between 17 and 59 months after UCBT	[103]
Diamond-blackfan anaemia	1	Successful seroconversion to vaccines (diphtheria, pertussis, tetanus, rubella, measles and BCG) administered 22–34 months post transplant	[104]
Miscellaneous			
Severe chronic active Epstein Barr virus	1	Complete remission without circulating EBV-DNA continued for 15 months post transplant	[105]
Behcet's disease	1	23 months post UCBT, the patient was doing well, and no signs or symptoms were noted	[106]

Adapted from Riordan [110]
Patient numbers noted from the article cited, but, in many cases, the total number of patients treated in other centres is much higher

of the cord blood clinical studies on non-malignant diseases. Examples provided are by no means exhaustive, but demonstrate the remarkable advances that have been made in the area of cord blood stem cell engineering and reenforce the fact that stem cells hold tremendous promise for the future of regenerative medicine and cellular therapy.

Chronic diseases such as diabetes mellitus, stroke, ischemic heart disease, haemoglobinopathies and chronic liver cirrhosis are social repercussions that have a huge impact on the health care system. They are a global priority of health and demand definite attention. The statistical incidence and prevalence of some of these chronic disease conditions is shocking. The WHO calculated that 20 million people worldwide have cirrhosis of the liver and/or liver cancer, arising predominantly among the estimated 500 million people (nearly 10 % of the world population) who suffer from hepatitis B (HBV) and hepatitis C (HCV) viral infections. Each year an estimated two million people die of liver disease (www.hepalfe.com/images/chronic liver disease) [93].

In the present day the only management available for such chronic diseases is supportive or palliative therapy ultimately demanding cell transplantation for cure in some cases, beta cell transplantation in the case of diabetes, bone marrow transplantation for thalassemia and hepatocyte transplantation for liver cirrhosis. However, the scarcity of available donors and the complications associated with the operative procedure justify the requirement for alternative treatment options for these illnesses. Cord blood stem cells could prove to have potential in developing cellular therapy for treatment of these and other similar disease conditions.

Liver Disease: Global Health Burden

Liver disease is highly prevalent worldwide. The World Health Organization estimated (2006) that approximately 20 million people worldwide with cirrhotic/mitotic indications arose mostly from 500 million (@ 8–10 % world population) victims of hepatitis B (HBV) or hepatitis C (HCV) viral infections [93]. Hepatic failure alone accounts for one to two million deaths per annum and is nominated the fifth leading cause of death around the globe. Decompensated liver function and ultimate failure has many culprits including: excessive alcohol consumption, aggressive forms of fatty liver disease, fibrosis, inflammatory liver conditions and unregulated ingestion of common over-the-counter medication, such as acetaminophen [93]. Within the UK alone, acetaminophen overdose contributes to 48 % of poisoning admissions to hospitals and is involved in an estimated 100–200 deaths per year [111].

Mechanism of Liver Damage and Ultimate Fate

The liver is an exceptional organ; in an event of parenchymal cell loss, the mammalian liver can cite at least three apparently distinct cell lineages to contribute to regeneration and repair after damage (Fig. 22.4). The first line of defence is provided by hepatoblasts/hepatocytes – themselves believed to be functional ‘stem cells of the liver’ [112, 113]. More severe liver damage calls upon activation of a potential stem cell compartment located within the intrahepatic biliary tree, giving rise to cords of bipotential cells that ultimately differentiate to hepatic or biliary epithelial cells. These hepatic stem/progenitor cells are referred to by different names including ‘oval cells’ and ‘small hepatocyte-like progenitors’ (SHPCs) in rodents [114, 115] and ‘intermediate cells’ in humans [115, 116]. This cell population has been shown to have ‘bipotential characteristics’ expressing morphological and immunophenotypic features typical of both hepatic and biliary epithelial cells. Wilson and Leduc, 1958, were the first to describe activation of this ‘reserve cell compartment’, which in humans is described as ‘ductular activation’ or ‘progenitor cell activation’ [117]. A third population of stem cells with hepatic potential resides outside the liver in the bone marrow. These haematopoietic stem cells may contribute to the low renewal rate of hepatocytes, but in the face of severe and/or extensive damage can contribute significantly to regeneration under very strong positive selective pressure [118–122].

Under conditions where the insult to the liver is extensive and/or chronic, the liver is crippled by hepatocyte senescence due to telomere shortening that is associated with ongoing proliferation during the prolonged chronic history of disease. During incidents where the intrinsic proliferative and clonogenic capacity of inherent hepatocytes/hepatoblasts is affected, extrinsic stem cells with hepatogenic potential are chemoattracted towards to the site of liver injury, finding a niche and attempt to salvage the liver. It should be noted, however, that in the diseased liver, there may be lack of the major growth stimulus and/or absence of substantial growth advantage for haematopoietic stem cells to correct and repair the damaged tissue. Another important factor is the intrinsic genetic regulation of stem cells [123]. In addition, there are many other regulatory systems involved, which dictate the replacement of damaged, aged or diseased tissue with new cells. Stem cells secrete factors which act through a paracrine system and play a role in regulating tissue regeneration. Systemic factors are also important in modulating signalling pathways critical to activate tissue-specific stem cells [124]. Liver cells (hepatocytes, cholangiocytes and progenitor cells), mesenchymal cells (Kupffer cells, endothelial cells and hepatic stellate cells) and the liver stroma collectively form the liver ‘stem cell

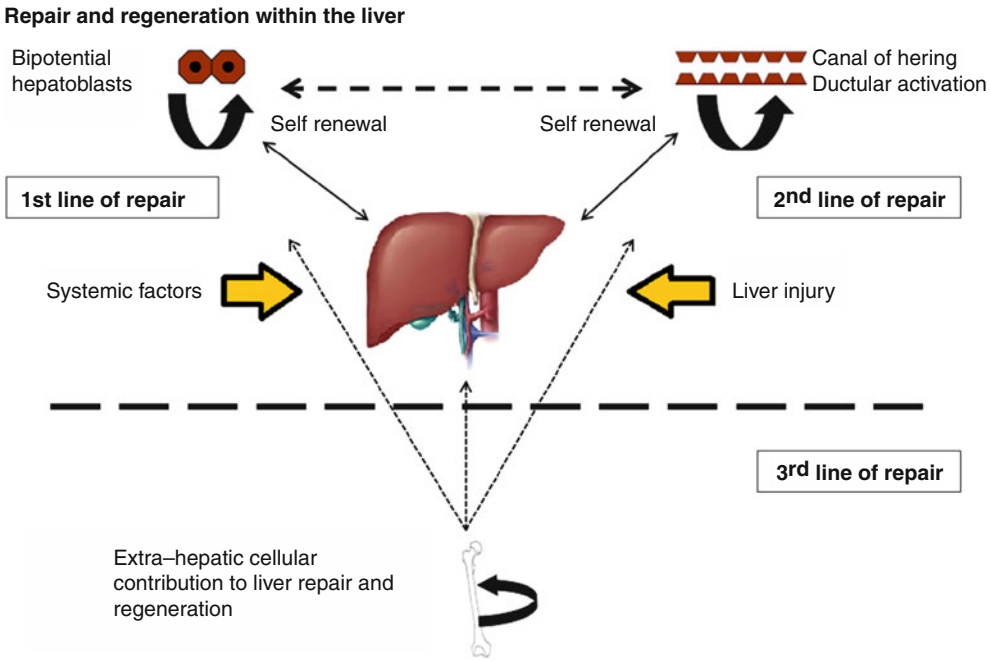

Fig. 22.4 Three different cell lineages that contribute to liver regeneration and repair after injury. First line of defence in the face of injury is provided by bipotential hepatoblasts which are capable of differentiating into hepatocytes and cholangiocytes. These are believed to be functional stem cells of the liver and are capable of self-renewal after loss. When hepatocyte renewal is compromised, or the liver damage is more severe, this calls upon activation of bipotential stem/progenitor cells in the canal of Hering, located in the intrahepatic biliary tree that take over the burden of regeneration. Under conditions where the insult to the liver is extensive and/or chronic or the intrinsic proliferative capacity of the liver is compromised, extrinsic stem cells from the bone marrow harbour to the site of injury and provide a third line of repair and regeneration. It should be noted, however, that systemic factors also play a pivotal role in modulating signalling pathways critical to tissue regeneration

niche' that regulates stem cell proliferation, maintenance and cell fate decisions. The importance of such cellular and noncellular interactions was demonstrated by Kon et al., who elegantly illustrated in vitro differentiation of small rat liver progenitor cells into hepatocytes upon coculture with non-parenchymal supporting cells [125]. Additionally, the fact that hepatocytes, which have a great growth potential in vivo, when isolated and put in culture, pose a huge challenge to maintain alive and differentiated also further reenforces the importance of such niches [126]. Even if stem cells did tend to retain much of their intrinsic proliferative potential when old, age-related changes in the systemic environment and niche in which stem cells reside preclude full activation of these cells for productive tissue regeneration.

Current Mode of Management of Liver Disease: Restrictions and Limitations

Despite worldwide prevalence and significant morbidity and mortality associated with severe liver disease, medical advancement so far often only allows successful treatment of compromised liver function through resection and transplantation surgeries [127, 128]. This is an invasive procedure and is limited by the availability of donor organs. Less than 30 % of patients on a waiting list receive a transplant [129, 130]. Non-treatable and/or incurable liver conditions impending transplantation are subject to supportive and palliative modes of management. These comprise non-biological and biological liver support systems. These supportive modalities have been introduced to standard therapy since the 1950s, to bridge patients awaiting organ transplant.

Non-biological or Artificial Liver Support Systems

Non-biological systems primarily provide detoxification/purification by removing toxins of hepatic failure. The repertoires of known substances that accumulate in the blood in hepatic failure aggravate injury to the liver. These substances inhibit the natural ability of the liver parenchyma to regenerate and simultaneously suppress the ability of residual hepatocytes to perform organ-specific functions (sick cell syndrome). Additionally, these substances cause neurological abnormalities and failure of the immune system and other organs (kidney and lung). These substances include not only small molecular weight toxins (ammonia, phenols, false neurotransmitters, free bile acids, etc.) but also mediators of

inflammation (cytokines, chemokines, anaphylatoxins, etc.). Vasoactive substances, endotoxins, cell growth inhibitors (e.g. TGFβ1) and other known and currently unknown harmful substances are also included [93]. Some of the latest advancements in blood purification technologies include: MARS® (molecular adsorbent recycling system), SPAD (single pass albumin dialysis) and SEPET (selective plasma filtration), but their utility is limited by their inability to provide some other missing liver functions.

Bioartificial Livers (BAL): Hepatocyte-Based Extracorporeal Devices

Bioartificial or hepatocyte-based devices represent advancement in the management of hepatic disorders. These cellular-dependent (animal or human cells) systems are able to come closer to providing 'whole liver' functions, including detoxification, biosynthesis and biotransformation. However, it is worth noting that in addition to the risk of xenosis in the animal cell-based systems (mainly of porcine origin), additional well-conducted studies are warranted to better demonstrate safety and proof of concept of these devices [93].

Thus far, development of an effective liver assist technology has proven challenging because of the complexity of liver functions that must be replaced, as well as the heterogeneity of the patient population.

Alternatively cellular therapies including hepatocyte transplantation can be used either to replace or increase the number of functional hepatocytes. The current source of hepatocytes is from discarded livers not suitable for whole organ transplantation, yet again limiting its accessibility. These cells are also used to establish primary cultures of hepatocytes, but have been hindered by their short lifespan and the rapid loss of hepatic function under in vitro conditions. There is still a great need for new sources of stem/progenitor cells with an ability to differentiate into functional liver cells. Isolation of an expandable population of adult human pluripotent stem cells will be an attractive alternative for current therapies. Before that, however, we need to better understand the cellular and molecular (genetic and epigenetic) mechanisms responsible for liver cell differentiation. Therefore, adequate in vitro platforms and animal models of liver disease are of great importance.

Cellular Therapy of Liver Disease: Regenerative Medicine, an Alternative Approach in Health Care

With liver transplantation currently the only successful treatment for acute hepatic failure or end-stage liver disease and the major limitation of donor shortage, considerable attention has recently been focused on identifying alternative modes of management for fatal liver disorders with regenerative medicine occupying the limelight.

Hepatocyte Transplantation

Hepatocyte transplantation was believed to be a potential solution with several reports demonstrated in animal models in rescue from lethal hepatic failure post primary hepatocyte transplantation. This was achieved through efficacious donor chimerism [131–134]. Two clinical trials previously demonstrated the effectiveness of hepatocyte transplantation for treatment of liver-based congenital metabolic disorders. Fox et al. transplanted allogeneic hepatocytes into the liver of a patient with Crigler-Najjar syndrome type I [135]. The patient survived for 11 months with partially corrected hyperbilirubinemia. Additionally, Dhawan et al. reported transplantation of cryopreserved hepatocytes into patients with inherited factor VII deficiency resulting in gradual reduction in their requirement for recombinant factor VIIa until after 8–10 weeks, requiring only 20 % of their original dose [136]. It is still questionable, however, whether hepatocyte transplantation can contribute to the rescue of liver-based metabolic disorders over the long term. Furthermore, ex vivo expansion of mature hepatocytes is not yet feasible because long-term cultivation of hepatocytes is associated with hypofunction of hepatocyte metabolism. It would, therefore, be greatly valuable if functional hepatocytes could be generated from stem cells, which could be propagated in vitro.

Foetal Liver Progenitor Cells

Research into the role of stem cell application in liver disease has, unfortunately, been subject to various limitations (Table 22.2). Liver progenitor cells identified in embryonic and foetal livers have been successfully differentiated into hepatocyte or bile epithelial cells [137–139]. Such studies have been restricted by the low yield of cells and the inadequate in vitro expansion after isolation [140]. Sandhu et al. and Oertel et al. both demonstrated in rat models that foetal hepatic stem/progenitor cells exhibited potency for reconstitution of adult liver but only under a particular set of conditions [141, 142]. Thus, it still remains unclear whether adult hepatic stem cells or progenitor cells can reconstitute recipient livers not subjected to genetic modification. To answer this question, therapeutic transplantation of hepatic stem cells must be carried out using minimal pretreatment and without genetic modification of the recipients. To overcome the problem of low cell yield, attempts were made by several groups to immortalise hepatocytes using viruses [143, 144] or the enzyme telomerase [145]. These approaches allowed expansion of hepatocytes but were compromised by

Table 22.2 Limitations of various stem/progenitor cells with potential for management of liver disease

Embryonic/foetal liver progenitor cells	Immortalised hepatocytes viruses/enzyme (telomerase)	Embryonic stem cells	Induced pluripotent stem cells	Adult stem cells bone marrow/peripheral blood
(a) Low yield of cells (b) Inadequate in vitro expansion [166]	(a) Compromised by phenotypic changes and karyotype abnormality over prolonged culture durations [167]	(a) Problems of genetic instability (b) High risk if teratoma (c) Ethical issues (d) Difficulties to produce large quantities of homogenous cells/tissue (e) Complications associated with feeder layers [35, 166]	(a) Genetic instability (b) Low efficiency of cell production [167]	(a) Invasive collection procedures (b) Differentiation potential widely studied, but proponents question the very existence of the process claiming that cell fusion is responsible for the phenomena [168]

phenotypic changes and karyotypic abnormalities over prolonged culture durations [146].

Embryonic Stem Cells

The introduction of embryonic stem cells (ESC) provided another potential source of human hepatocytes. Mouse embryonic stem cells (mESCs) have been shown to be able to differentiate down the hepatocyte lineage which can integrate into liver tissue and produce albumin [147–150]. Similar work has been demonstrated in human embryonic stem cells (hESCs) as well [151, 152]. Although ESCs have proliferation and differentiation properties, they cannot be used in the clinic because of their genetic instability and a high risk of tumour formation. ESCs are also limited by ethical issues surrounding the use of human embryos associated with the fact that their retrieval requires destruction of an embryo. The scientific realism surrounding ESCs is that the production of large quantities of homogenous cells/tissue for clinical application is difficult, and there could be potential complications associated with the animal feeder layers on which human ES cells tend to rely on in addition to the risk of teratomas. Hence it is unlikely that embryonic stem cell (ESC)-derived treatments will be available for clinical use anytime soon.

Induced Pluripotent Stem Cells

Recently developed techniques by Takahashi and Yamanaka for pluripotency induced in adult fibroblast give an interesting alternative for ESC [153]. These induced pluripotent stem cells can be generated without ethical concerns, but their genome instability and low efficiency of cell production raise the same concerns as for ESCs when considering their clinical use.

Adult Stem Cells

Adult stem cells (predominantly bone marrow stem cells) have been extensively investigated as a potential source of liver stem cells and as a means to regenerate the diseased liver. This attention was fuelled by the identification of a population of liver stem cells in rodent (oval cells) that expressed HSC markers (e.g. Thy-15). And, hence, it was postulated that these cells most probably originated from the bone marrow [120]. Subsequently, BM transplants in animal models of liver disease were carried out, and analysis suggested that BM could contribute to a mature hepatocyte population [118, 121]. These exciting results lead to further investigation of this resourceful stem cell population, but the results obtained were contradictory with little evidence for a significant repopulation of the liver parenchyma by BM-derived cells [154–157]. Additionally, proponents suggested that any rescue resulting from BM transplantation was the result of cell fusion of the BM-derived cells with the diseased hepatocytes, resulting in a form of cellular gene therapy. Furthermore, it was suggested that the unique selection pressure seen in these animal models triggered the fused cells to divide to repopulate the liver [158]. It is worth noting that a selection pressure of this magnitude is unlikely to be available for most clinical applications.

Mesenchymal stem cells (MSCs) derived from various tissues including bone, fat and dental tissue have also been explored as an alternative source of donor cells. Animal studies have previously demonstrated the ability of human adipose tissue MSC-derived hepatocytes to restore some liver function in nude mice with acute liver failure [159]. Additionally, van Poll et al. showed that systemic infusion of soluble factors secreted from MSCs provided a survival benefit and prevented the release of liver injury biomarkers [160]. Thus it may be argued that the transplanted MSCs did not play a crucial role in reconstitution of the recipient liver, but instead, the soluble factors that they secreted were liver protective. Additionally, contradictory results have demonstrated that MSCs do not differentiate into hepatocytes in rat livers [161]. Such apparently contradictory results have been commonly seen in this field and probably reflect a combination of factors, including the use of varying cell derivation and differentiation protocols and the fact that the models of liver injury are often differing and rarely repeated. Although more in vivo research is definitely needed to address these issues, MSCs do appear to ameliorate liver injury in some

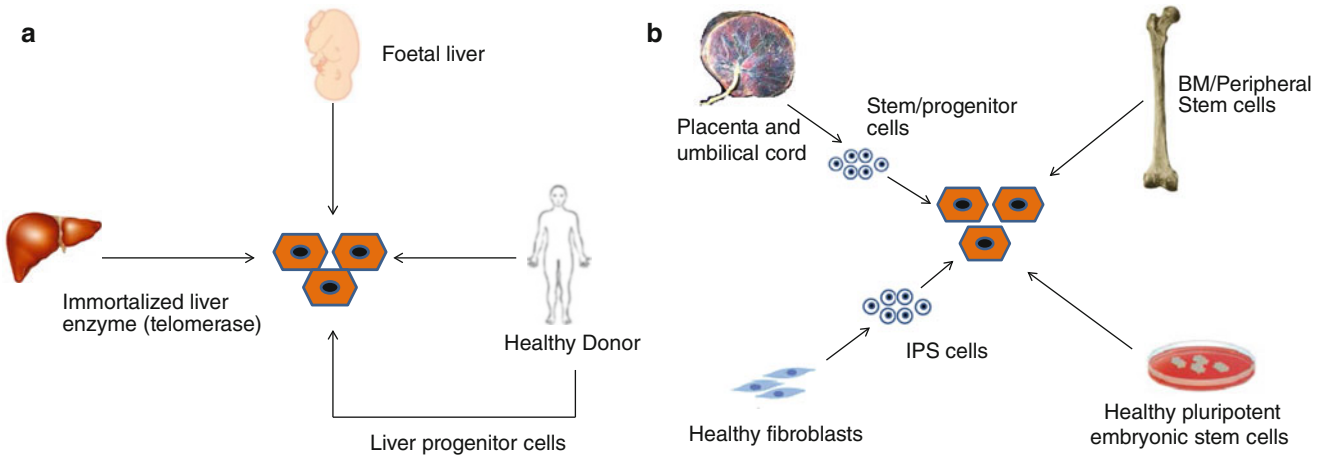

Fig. 22.5 (**a**) Hepatocyte sources for cellular therapies for in vitro modelling of liver disease. Ex vivo adult hepatocytes can be isolated from healthy donor livers, yet these cells are scarce due to donor shortage and upon isolation have a limited proliferation capacity and lose functionality. Alternatively, adult and foetal liver progenitor cells can be extracted from healthy donors and in vitro directed hepatocyte differentiation induced. (**b**) Hepatocyte sources for cellular therapies for in vitro modelling of liver disease. Human embryonic stem cells derived from healthy blastocyst undergo expansion and differentiation into hepatocyte-like cells by employing extracellular signals to mimic human liver development. Human induced pluripotent stem (*IPS*) cells can be generated by reprogramming healthy somatic fibroblast cells from healthy donors followed by induction of hepatocyte-like cell differentiation. Both the above sources of hepatocytes are associated with complications in terms of clinical applicability. A much safer and more resourceful alternative are the stem/progenitor cells extracted from the umbilical cord and cord blood which can be exploited in in vitro hepatocyte differentiation systems providing an ideal model to study liver development and offer a valuable tool for cellular therapy of liver diseases

way or the other. Moreover, it may be that both the exact derivation and preconditioning of the starting cells and the host liver environment will determine the resulting phenotype of the transplanted cells.

The clinical studies reported for BM therapy for liver disease involve infusion of the enriched haematopoietic cell fraction ($CD133^+$, $CD34^+$ or monocytes) [162–164]. Interestingly, the majority of in vitro and in vivo experimental work suggests that the MSC population in BM are inducible into hepatocyte-like phenotype, and there is very few data suggestive that HSCs have a similar potential. The logic, however, for using the haematopoietic cell fraction in cirrhotic liver conditions may be due to the modulating and remodelling effect of the monocyte-macrophage population on scar tissue [165]. As pointed out, the results achieved from ADS research as an alternative cell source for the management of liver disease has been exciting but has also simultaneously highlighted many grey areas that need further investigations.

A novel stem cell source that has been explored more recently is the umbilical cord and cord blood. This stem/progenitor cell reserve provides a very abundant and valuable resource that still remains to be exploited therapeutically in the management of liver injury and disease.

As far as cell therapy of end-stage liver disease is concerned, it may be safe to conclude that stem/progenitor cells that can be efficiently differentiated into a hepatocyte phenotype can be used for ex vivo purposes, such as liver support devices, toxicology studies and drug testing. For transplantation procedures, however, it is essential to ensure that the cells infused are preprogrammed to either differentiate into or remain as hepatocytes in vivo post transplantation, and research thus far is some way short of this mark. Additionally, it is worth considering what the best treatment approach would be. Would it prove more fruitful to transplant hepatocyte-like cells in the extremely harsh environment of a cirrhotic liver, where endogenous professional hepatocytes find it hard to survive or to modify the cellular and extracellular milieu, to allow the endogenous hepatocytes and progenitor cells to regenerate the liver? The latter may well be a more realistic medium-term goal for cell therapy (Fig. 22.5).

Hepatic Differentiation Properties of Adult Stem Cells

Work done on adult stem cells is in close context of ageing, cell repair strategies and haematopoiesis. Previous studies have shown the possibility of turning bone marrow (BM) and umbilical cord blood (UCB) stem cells into hepatic-like cells in vivo and in vitro [13, 34, 35, 75, 151, 152, 170–174]. The ability of bone marrow to contribute to hepatocytes was first demonstrated by Petersen et al., who showed that bone marrow cells transplanted into lethally irradiated mice engrafted in the recipient's liver and differentiated into liver stem cells (called oval cells in mice) or mature hepatocytes [120]. Soon after, Alison et al. and Theise et al. also illustrated in rodents and in humans that hepatobiliary cells could be derived from bone marrow [118, 121, 122]. With Y-chromosome staining and liver-specific markers, they detected bone marrow-derived hepatocytes in livers of irradiated mice and humans after gender-mismatched bone marrow transplantation.

Gordon et al. reported that human CD34+ cells mobilised in peripheral blood by administration of G-CSF (granulocyte colony-stimulating factor) followed by leukapheresis, and reinfusion of these cells in patients with liver insufficiency resulted in improved liver function in patients [175]. These interesting results, however, were not reproducible by several groups including Wagers et al., Kanazawa et al. and Cantz et al. [154, 176, 177]. Even when results were replicable by groups, as Lagasse et al. [178], they were noted not to be the result of direct differentiation but rather occurring due to fusion of haematopoietic stem cells with recipient hepatocytes in the animal models [179, 180].

Although the exact mechanism for the effect on liver function is not clear and little is known as to which biomolecular and biochemical pathways regulate such differentiation, such data may reflect activation of genes corresponding to a hepatocyte differentiation programme upon exposure to the injured liver environment.

Although proponents see ADS as an attractive alternative to the use of embryonic stem cells in regenerative medicine, opponents have questioned the very existence of the process claiming that cell fusion is probably responsible for the phenomenon. Several critics have challenged the concept of stem cell plasticity. Issues have included the inability to reproduce data, and the suggestion that some apparently reprogrammed ADS could be engrafted cells fusing with cells in their new location. This opinion is based on experiments exploring the outcome of co-culturing BM with highly volatile embryonic stem cells, but not ADS, and noting occasional tetraploid cells from the fusion of the two cell types [181, 182]. A better understanding of the mechanisms of lineage-specific differentiation and plasticity of pluripotent stem cells would provide critical clues for the use of stem cells in regenerative medicine. For this purpose the scientific community needs to develop reliable in vitro and animal models that will allow for better understanding of how to efficiently differentiate ADS and UCB stem cells into functional hepatic tissue. A new approach of liver tissue-engineered constructs in 3-dimensional environment in vitro gives a unique tool for preclinical and toxicological studying of ADS and UCB for their clinical applications in the future. Hence, as previously elaborated earlier it may be safely concluded that umbilical cord- and cord blood-derived stem cells offer multiple advantages over truly adult stem cells and over embryonic stem cells for liver differentiation.

Can Cord and Cord Blood Stem Cells Regenerate Liver Tissue?

Taking into consideration the superiority of umbilical cord blood stem cells over bone marrow-derived cells; several groups explored the potential of human umbilical cord blood to generate hepatocyte and biliary epithelial cells. These experiments were performed using mononucleated cells from cord blood for in vivo transplantation in animal models. In vivo studies were first performed in sheep by Almeida-Poroda et al. and then in rodents by Newsome et al., Wang et al. and Ishikawa et al., by transplanting cells into sublethally irradiated NOD-SCID mice [183–186]. Although these recent publications highlight the differentiation potential of human UCB cells and were able to produce hepatocytes, further characterisation of these differentiated cells was demanded. In 2005, Sharma and colleagues, for the first time, characterised these human UCB-derived hepatocyte-like cells after in vivo experiments in mouse animal models and demonstrated the ability of these cells to express human albumin- and human hepatocyte-specific antigen, Hep Par1 [187]. They compared BM cells with human UCB mononucleated cells in parallel controlled studies and showed that cord blood was superior to BM in its differentiation potential. It could have been argued that because neonatal UCB stem cells are different from BM stem cells and may have a reservoir of preformed hepatic progenitors, which may not be present in the BM preparation, they demonstrated better results [188, 189]. However, to avoid such questions, Sharma et al. performed the experiments using both adult and neonatal BM cells for transplantation but were unable to illustrate an increase in frequency of BM-derived hepatocytes in the model livers of neonatal BM transplantation [187].

Until 2005, all research done on human cord blood was performed using cord blood-derived mononucleated cells, and the studies were executed in animal models. Mononucleated cells constitute the entire white blood cell/leukocyte compartment of blood, of which stem cells are a component. Although UCB demonstrates a better tolerance for human leukocyte antigen (HLA) mismatch compared to ADS, perhaps due to the immaturity of its immune cells, the production of a purified population of stem cells from cord blood for transplantation would further reduce any risk of immune-related graft rejection. McGuckin et al. reported the world's first production of haematopoietic cells, expressing embryonic stem cell markers, from cord blood. These cells were termed cord blood-derived embryonic-like stem cells (CBEs) and were produced by exposing UCB to an immunomagnetic cell separation technology that allows sequential removal of nucleated granulocytes, haematopoietic myeloid/lymphoid progenitors and erythrocytes, leaving behind a purified population of very immune naive stem/progenitor cells [75]. Subsequently other reports concerning the existence of circulating embryonic stem cell-like cells during foetal development were generated [190, 191]. McGuckin et al. further demonstrated the differentiation potential of these cells and showed that these cells were capable of generating endodermal tissues, including hepatocyte-like cells in 2-dimensional and 3-dimensional

culture systems, expressing hepatocyte-specific markers [75] as well as pancreatic-like cells testing positive for insulin and C-peptide [192]. This was achieved by thoroughly studying classic liver biology and development and creating an artificial culture system that closely resembles the natural micro-environment that is needed for normal development of the liver. Similar protocols have been reported in human embryonic stem cell-derived hepatic cells but not in cord blood studies [151]. A few other research groups have also been able to successfully differentiate cord MSCs into hepato-biliary cells [34, 193]. Campard and colleagues are amongst the few research groups that demonstrated differentiation of cord matrix (Wharton's jelly) MSCs into hepatocyte-like cells [166].

It is worth noting that since the isolation MSCs from cord blood is inefficient, extraction of haematopoietic stem/progenitor cells may prove more resourceful when using cord blood; as irrespective of the size of the cord blood units, these cells can be isolated from nearly every cord blood specimen processed. The umbilical cord, on the other hand, is a brilliant resource for MSCs. However, one of the limitations of MSCs is their strict dependence on selected lots of foetal bovine serum that has limited clinical applicability of ex vivo-expanded MSCs. This drawback, however, may be overcome by restoring to low serum or serum-free culture systems as demonstrated by Reinisch et al., who for the first time demonstrated propagation of both BM and UCB MSCs in bovine serum-free systems using human platelet lysate-conditioned medium [194]. Both these aspects, (i) generation of artificial liver tissue in vitro and (ii) use of defined culture media for stem cell differentiation, are crucial for establishing a reliable in vitro platform for studying cellular and molecular mechanisms of stem cell differentiation towards the liver.

Liver Tissue Engineering

It is worth noting that most in vitro hepatic tissue models available for research and development use 2-dimensional culture systems. These systems fail to represent the physical cell-cell interactions of a 3-dimensional human liver and also do not always produce scientific data that can be fully translated to physiological interpretation. The quest for an efficient cellular technology that can be exploited for the management of end-stage liver diseases has paved the way for 3-dimensional tissue engineering. Such technology employs synthetic biodegradable porous scaffolds and rotational cell culture systems, also referred to as bioreactors, which allow cells to grow and differentiate in a 3-dimensional environment facilitating cell-cell interaction. Groups like Baharvand et al. have utilised scaffolds to effect 3-D hepatic differentiation of human embryonic stem cells [151], and McGuckin et al. have demonstrated similar results from umbilical cord blood by employing the microgravity bioreactors [75].

Clinical Trials

After several reports in animal models, Theise et al. were the first to confirm the ability of bone marrow to generate hepatocytes and cholangiocytes in humans by evaluating archival autopsy and biopsy liver specimens obtained from gender-mismatched therapeutic BM transplantations and from orthotopic liver transplantations [122]. Approximately 11 BM stem cell transplantation clinical trials for treatment of liver diseases in humans have been published thereafter [196]. These include work done by Mohamadnejad and colleagues in 2007, which carried out autologous bone marrow-enriched $CD34^+$ haematopoietic stem cell transplantation into hepatic arteries of four decompensated liver cirrhosis patients. Although a mild degree of improvement of liver function was noted in two out of four patients, one died due to radiocontrast nephropathy and hepatorenal failure. Thus they concluded that intra-hepatic artery is not a safe mode of stem cell transplantation but could not preclude the use of $CD34^+$ cells via other routes of administration [197]. Conversely, clinical trials performed on similar patients by Lyra et al. [164, 198] and Gordon et al. [175], using mononucleated cell-enriched BM cells, reported intra-hepatic artery route of infusion as safe and feasible. Mohamadnejad et al. further reported on the safety and feasibility of autologous bone marrow mesenchymal stem cell transplantation in patients with liver decompensation due to cirrhosis. This time, however, the stem cells were administered via a peripheral vein in four patients. No post-operative side effects were noted, and all patients showed evidence of improvements of liver function and a better quality of life [199]. The efficiency of intravenous infusion was confirmed by studies performed in mice by Kou et al. [200]. Terai et al. and Gordon et al. reported transplantation of autologous BM stem cells and human $CD34^+$ stem/progenitor cell populations mobilised into the blood by granulocyte colony-stimulating factor, respectively, and both noted improvement of liver functions in patients post transplantation [163, 175]. It seems clear that trials of BM stem cell treatment in patients with liver disease are still at a preliminary stage, and a better understanding of the physiology and mechanism of action of BM stem cell in animal models of liver disease is needed. The results of these clinical trials are, however, very exciting and open the roads for exploring the potential of umbilical cord and cord blood stem cells for cellular therapy of liver disease. In addition to intra-hepatic delivery of stem cells for treatment of liver disease, tissue engineering offers novel opportunities for the generation of extracorporeal liver devices. Such technology

would allow temporary replacement of liver function buying time till a suitable organ is available for transplantation.

Thus far the research done into exploring the potential of the umbilical cord and cord blood for regenerative management of end-stage disease is commendable. Although the outcome of the various studies highlights extra-embryonic tissues as an indispensable reserve with immense potential for liver therapy, limitations such as lack of consensus in immunophenotype of liver progenitor cells, uncertainty of the physiological role of reported candidate stem/progenitor cells, long-term efficacy and safety challenge the use of these cells in humans. Current molecular techniques of stem cell identification are confounded by cell fusion, horizontal gene transfer, incomplete differentiation and chimera formation. It is exciting to note that stem cell transplantation and phase 1 trials of bone marrow transplantation in humans for liver diseases [197, 199] are underway but require more robust verification. More research is definitely demanded to help identity the best source of stem/progenitor cells that can transferred from the bench to the bedside for the management of patients with severe or life-threatening liver disorders.

Cord Blood Banking

Scientific data generated from research into the pluripotent nature of UCB-derived stem cells greatly highlights the need and importance of cord blood banking.

Banking is the process whereby UCB stem cells are saved for potential medical uses in the future. The procedure includes the process of cord blood collection from the umbilical cord which can be performed in utero (during third stage of labour, prior to delivery of the placenta) or ex utero (after the umbilical cord has been clamped and completely detached from the baby). Both procedures can yield similar amounts of cord blood (on average between 70 and 120 ml per unit). It is encouraged by the authors and other experts that unless there is a very strong medical indication otherwise, that only ex utero collection is warranted since the procedure poses no harm in any way to the mother or the baby. It is a completely non-invasive process. The blood collected is then subjected to plasma depletion and removal of red blood cell compartment and is tested to determine whether it meets the eligibility standards. The processed cord blood unit (buffy cells) which contains, in very low quantity, the stem cells is then cryopreserved by freezing in liquid nitrogen at −196 °C with a cryopreservative mixture. This enables cellular ageing and damage to be reduced.

Thus far, three main types of cord blood banks exist:

(i) Public cord blood banks storing anonymised units that can be released to any patients with a partial or total HLA match
(ii) Private ‘family’ banks which, against a fee, store units for exclusive use by the family paying for the service
(iii) Mixed banks, offering both public and private services

Public banks have come into existence in many countries since the early 1990s. These banks usually meet strict quality control criteria and national/international accreditation, but are focussed almost completely on allogeneic haematopoietic transplantation only, since in most diseases of the bone marrow and immune system, the treatment requires replacement, rather than regeneration. The rationale behind public banking is that they offer the advantage of having the sample already stored in a bank, and if there is a match, the unit can be shipped to the hospital immediately. On the other hand, bone marrow registries are subject to complexity of HLA and immunological matching and other logistical complications that hinder the timely localisation of a potential bone marrow donor. However, when compared to a world registry exceeding 11 million bone marrow donors, only estimates of 700,000 cord blood units are stored in approximately 50 cord blood banks in about 20 countries.

Private cord blood banks charge a fee to store umbilical cord blood samples for about 20–25 years. It has been estimated that in excess of 1.1 million units are stored in private banks worldwide, including >500,000 in the USA and just over >300,000 in Europe. The two main issues associated with private banking are (i) that not enough private cord blood banks are complying with regulations and accreditations for processing quality control and (ii) that the use of private cord blood units is more likely to be used for autologous therapies, which are still at the early stages of development. So there is a rigorous need for strict regulatory frameworks to better prevent unreliable service. Additionally, some cord blood banks (public and private both) are struggling financially, so it is of critical importance to find the most effective and economical processing and storage methods [201, 202]. Many factors affect the success of processing and the yield of stem cells generated [203], so it remains an important priority to find the best possible methods of cord blood separation that will allow even cord blood units of small volumes to prove clinically viable.

More recently, in addition to cord blood, cryopreservation of the umbilical cord has also been made available in some countries. The focus of such cord tissue banks lays in cell therapy applications of MSCs for regenerative therapy in cell transplantation.

If in future liver regenerative therapies are going to be useful, then an availability of tissues will be key to the success. Umbilical cord and cord blood, stored in banks, may be able to provide that, not least to the children whose samples have been stored since birth. Since hospitalisations of children following toxic ingestion continue to be a problem, availability of a regenerative cell source is something that needs to be considered.

Conclusion and Future Advancements

The first clinical application of UCB for treatment of acute lymphoblastic leukaemia in 1970 fuelled a lot of interest in cord blood as a valuable stem cell resource. Twenty years later, the value of cord blood was internationally appreciated, and the concept of cord blood banks came into existence in 1990.

The scarcity of donor livers and other organs is a driving force behind the emerging field of tissue engineering and regenerative medicine. Efforts to build tissues from the ground up have proven very challenging and have yet to be translated into transplantable replacement organs. The liver, in particular, poses a great challenge for this task. Each hepatocyte functions as a metabolic factory demanding constant, direct contact with the vascular system. One of the limitations of artificial in vitro tissue engineering so far has been the lack of an established vascular system associated with the cultured cells [169, 195]. Powerful developments in the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies. A latest advancement in the field of transplantational medicine has developed a technique that may someday translate into growth of transplantable replacement organs. In 2008, Ott et al. performed decellularisation of rat hearts leaving behind an intact vascular system that facilitated repopulation of the structural matrix and subsequent survival and function of introduced cardiac and endothelial cells [204]. This technique was refined in 2010 and applied to rat liver [205]. This work is a breakthrough in the field of regenerative therapy. Decellularised liver matrix may provide a biocompatible natural scaffold on which stem/progenitor cells can be reseeded. Having the detailed microvasculature of the liver is a major advantage to growing liver tissue in a synthetic environment. These results provide a proof of principle for the generation of transplantable liver graft as a potential treatment for liver disease. It must, however, be emphasised that although they are very promising advancements in the field of liver regeneration, we are still a long way from translating research into clinical application. In vitro-developed bioengineered liver tissue constructs will need to be tested in animal models of liver disease with detailed critical evaluation of subsequent results to determine their complete potential. Only then should such research be translated to clinical trials in humans.

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Crushed Finger and Its Repair After Placing It Inside an Abdominal Fatty Tunnel for 6 Weeks: A Preliminary Experience Report

23

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Introduction

Crushed and/or smashed finger injury is very commonly seen in the emergency of any busy government hospital in Calcutta, India. The cause may be due to a closing door, a hammer, or a heavy object; sometimes this could be the result of a cut with a knife or power tool such as a saw, sander, lawnmower, or any other machine tool used at home or in industry. In addition, finger bite injury is also seen at times as a last attempt of resistance to prevent rape, and there have also been cases of partially or totally severed fingers brought to the hospital emergency – the injury being caused by wives of a minority community fighting for familial supremacy. Generally in minor cases, the commonly injured components may include the skin, bone, nail, nail bed, tendon, pulp, and padded area of the fingertip. Everything depends upon the force applied to the area, the angle of application condition, and the movement of the finger at that point of time. The skin on the palm side of the fingertips is very specialized in that it has many more nerve endings than most other parts of the body. These nerve endings enable the fine sensation of the fingertips, and these can also be damaged. When this specialized skin is injured, an exact replacement may be difficult.

Severe crush or avulsion injuries can completely remove some or all of the tissue at the fingertip. If just the skin is removed and the defect is less than a centimetre in diameter, it is often possible to treat this injury with simple dressing changes. If there is a little bit of bone exposed at the tip, it can sometimes be trimmed back slightly and treated with dressings as well. For larger skin defects, skin grafting is occasionally recommended. Smaller grafts can be obtained from the little finger side of the hand. Larger grafts may be harvested from the forearm or groin. When patients lose more than the skin and have exposed bone, the injury may need to be covered with a flap of skin that has some soft tissue attached to it for padding. Small wounds on the tip of the finger may be covered with a flap from the injured finger. Larger wounds, such as those that result in substantial loss of the pulp, require a flap that is elevated from an adjacent finger or other source. The flap remains attached to its original site so that it has sufficient blood supply while it is stitched to the finger wound. A skin graft is used to cover the donor site defect. After a few weeks, the flap has enough blood supply coming from the injured finger as it heals into its new location and can be divided from its origin and fully set into the wound. Mobility of the tip is also checked, as injuries can occur to the tendons that bend or straighten the fingertip. X-rays are often needed to see if the bone has been injured.

Fractures of the bone in the tip of the finger are common. Very small fractures of the end or tuft of the bone usually do not affect the strength of the bone. Repair of the soft tissue, such as the nail bed, usually realigns and stabilizes these bone fragments. Fractures closer to the joint may require a splint or even a temporary metal pin(s) to hold the bone fragments in proper position. Some swelling is expected. If any of the fingers or parts of the hand seem severely swollen and bent out of shape and there is extreme pain, then there is probably a broken bone. If the damage is too severe,

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amputation of the fingertip may be necessary. Fingertip sensitivity is common and may last for many months. Sometimes the feeling in the fingertip is limited. The contour may have some distortion, and the quality and texture of the skin may be different from the very specialized skin that normally covers the fingertip. There also may be some deformity at the donor site of a graft or flap. Stiffness can be a concern, especially if a flap is needed.

The present paper reports a novel method that a team of doctors in Kolkata (earlier Calcutta), India, have used to treat finger injuries.

Materials and Methods

Patients admitted to the emergency of Vidyasagar State General Hospital, Calcutta, India, with crushed injury of the finger were recruited as subjects for the present study between October 2009 and September 2010 after taking necessary medicolegal special consent from the patient and the permission of the Ethical Committee of the Institution.

Review of Existing Literature

Many attempts to improve vascularity and prevent amputation have been recorded, for instance, the use of a regional first web flap to cover the amputated stump of the thumb [1], in case of severe crushing injury involving the thumb. Further, one-stage osteoplastic reconstruction of the thumb has been attempted based on a reverse flow of the forearm osteocutaneous flap. This is indicated in traumatic loss of the thumb at or around the metacarpophalangeal joint level [2]. In some cases of mutilating hand injuries resulting in bone and soft tissue defects, osteocutaneous flaps can be used for osteoplastic thumb reconstruction as a definitive procedure or followed by toe transfer [3]. Since the popularization of microvascular toe transfer, a tendency to relegate osteoplastic reconstruction techniques for the thumb has been reported [4]. Moreover, there are nonmicrosurgical methods as well like the nonmicrosurgical reconstruction of crushed and avulsed amputations of the distal thumb [5].

The introduction of supermicrosurgery, which allows the anastomosis of smaller calibre vessels and microvascular dissection of vessels ranging from 0.3 to 0.8 mm in diameter, has led to the development of new reconstructive techniques. New applications of this technique are for crushed fingertip replantations with venule grafts and toe tip transfers for fingertip loss [6]. In addition, topical irrigation with urokinase and low-molecular-weight heparin or enoxaparin solution has been reported to significantly reduce thrombosis rate at the anastomosis site of the crushed arteries in clinical practice without uncontrolled adverse effects [7]. In case of total loss of blood supply of the affected finger, "piggyback" techniques for finger transfer may be used which simplifies, with a high success rate, the hazardous second-stage transfer. In this method after a successful ectopic transplantation, the transplanted part is transferred to the original site not as a free tissue transfer but as a pedicle flap in which the transplanted finger is piggybacked to the groin flap and then the composite tissue (groin and finger) is transferred to the stump. After a waiting period of 3–4 weeks, the flap is severed sequentially or in one setting. Graft take, complications, sensitivity, patient satisfaction, and demographic data of the patients were then evaluated. Graft take was 75 % in the first stage and 100 % in the second stage [8]. Postoperative seromas here have been recorded as a problem. Although drains are still commonly used during the initial postoperative period, certain modifications, suction undermining, and progressive tension sutures have been found to produce superior results [9].

The Kolkata group of doctors have devised a new surgical approach to this very common problem of crushed finger injury.

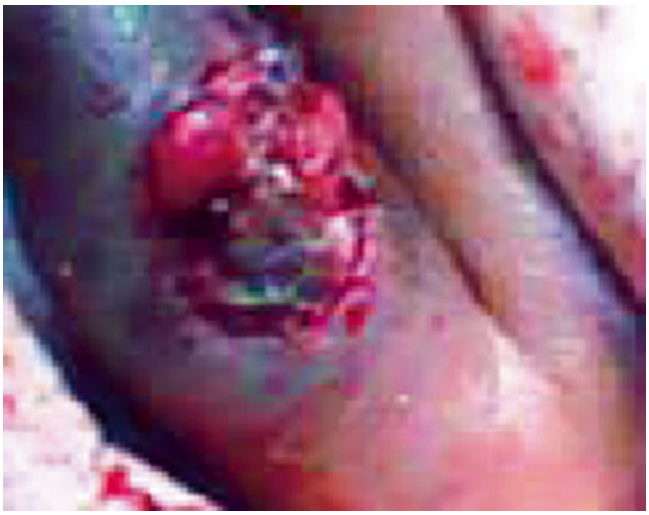
Result and Analysis

Finger surgery is a super-specialty even for qualified plastic surgeons. Reconstructive surgery today, however, is inundated with new ideas. Personnel with super-specialty training in plastic surgery are not easily available in Calcutta's free government hospitals except in postgraduate teaching hospitals. Such surgeons also practice in expensive tertiary care private hospitals. The present study was undertaken keeping in mind the paucity of plastic surgeons in free government hospitals and the expense involved in private hospitals as well as the large number of poor patients reporting with a variety of crushed finger injuries in government hospitals. Seven patients with age varying from 19 to 58 years (five females and two males) were enrolled for emergency surgery after necessary specialized consent at Vidyasagar Government Hospital between October 2009 and September 2010 (Table 23.1). The surgical technique used was very simple and may even be replicated in an emergency surgical set-up adjacent to the busy emergency admission department of any state general hospital. Doctors with basic surgical skill and understanding may perform this surgery with ease, and the results of this procedure are very promising and reassuring.

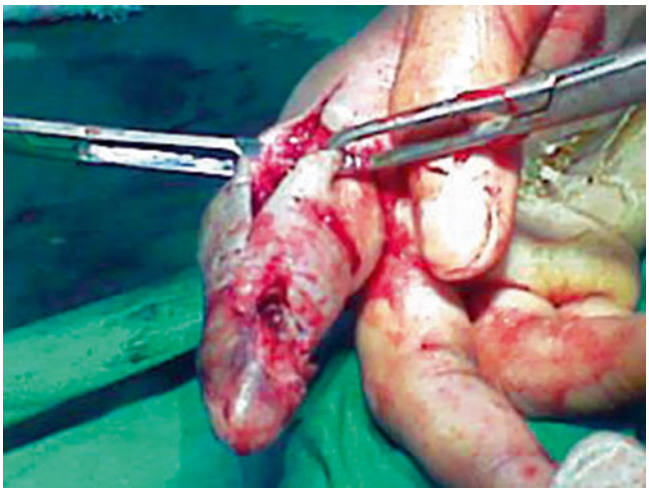
Photographs of the steps of the surgery for a typical crushed finger presentation are shown here (Photographs 23.1, 23.2, 23.3, 23.4, 23.5, 23.6, 23.7, 23.8, 23.9, 23.10 and 23.11). The typical finger with crushing injury and loss of skin and unexplained injury of the deeper structures like the

Table 23.1 Seven patients with age varying from 19 to 58 years (five females and two males) were enrolled for emergency surgery after necessary specialized consent at Vidyasagar Government Hospital between October 2009 and September 2010

Name	Age	Sex	Grade of injury	Type of injury	Procedure	Follow-up visit after 3 months
AKR	19	M	Crush injury	Skin + fat + blood vessel	Fatty tunnelling	Satisfactory
VD	52	F	Crush injury	Skin + fat + blood vessel	Fatty tunnelling	Satisfactory
SD	24	F	Crush injury	Skin + fat + blood vessel + bone	Fatty tunnelling	Satisfactory
JR	28	F	Crush injury	Skin + fat + blood vessel + bone	Fatty tunnelling	Satisfactory
PC	33	F	Crush injury	Skin + fat + blood vessel + bone	Fatty tunnelling	Satisfactory
JC	58	F	Crush injury with amputation	Skin + fat + blood vessel + tendon	Fatty tunnelling	Satisfactory
HM	39	M	Crush injury with amputation of finger tip (quasitotal)	Skin + fat + blood vessel + bone + tendon	Fatty tunnelling	Satisfactory

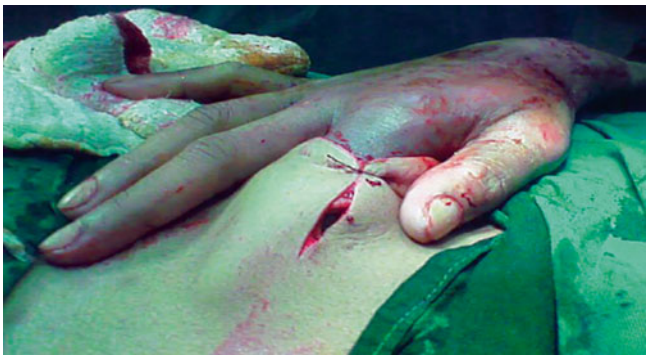


Photograph 23.1 Index finger inadvertently crushed between two mechanical tools

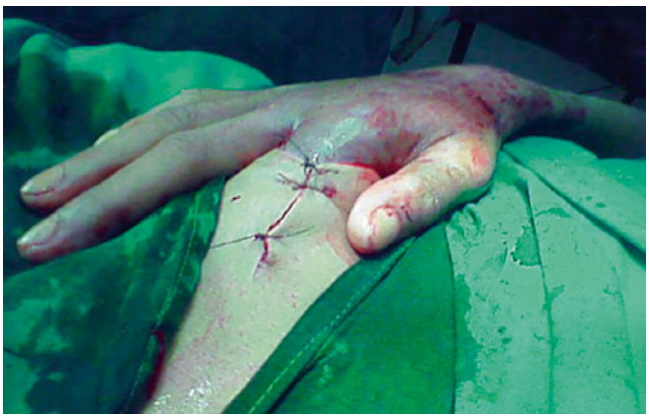


Photograph 23.2 Same index finger after a septic and antiseptic dressing in the O.T; a small incision has been made for drainage

joint, tendons, nerve, and fatty tissue was properly cleaned with normal saline followed by Betadine and spirit in the emergency, and the patient was sent to OT. After proper antiseptic dressing and draping in the OT, anaesthesia



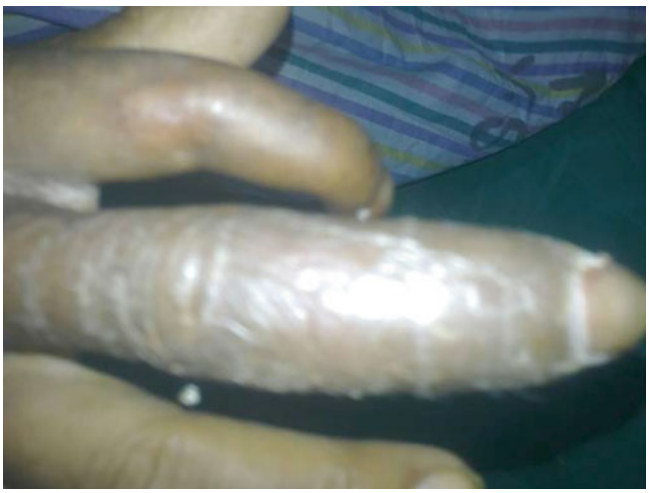
Photograph 23.3 Same index finger is placed inside a freshly created artificial abdominal tunnel in between two layers of fatty tissue



Photograph 23.4 Skin is stitched over the tunnel containing the index finger with interrupted silk stitches

(general) was given and a small 2 cm incision is made adjacent to the umbilicus. A tunnel is dissected by a curved artery forceps between the fascia of Camper and Scarpa, and the injured finger is pushed into the tunnel with fixation at the entry carefully by 3–4 (00) non absorbable suture (Vicryl) knots so that it is not displaced after its placement in the fatty tunnel (Photographs 23.1, 23.2, 23.3, and 23.4). In all cases, the finger is taken out after 6 weeks (Photographs 23.5, 23.6, 23.7, 23.8, 23.9, and 23.10). Photograph 23.11 is a typical cut of the finger involving the skin, bone, tendons, etc. before its placement in the fatty tunnel.

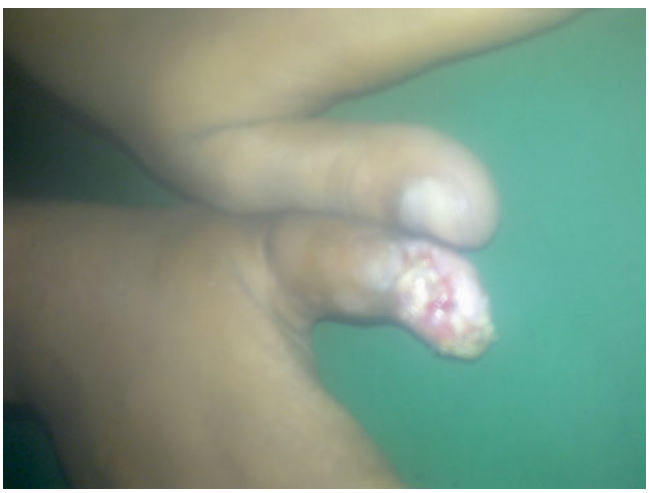
Photograph 23.5 Retrieved injured finger (middle) after 6 weeks



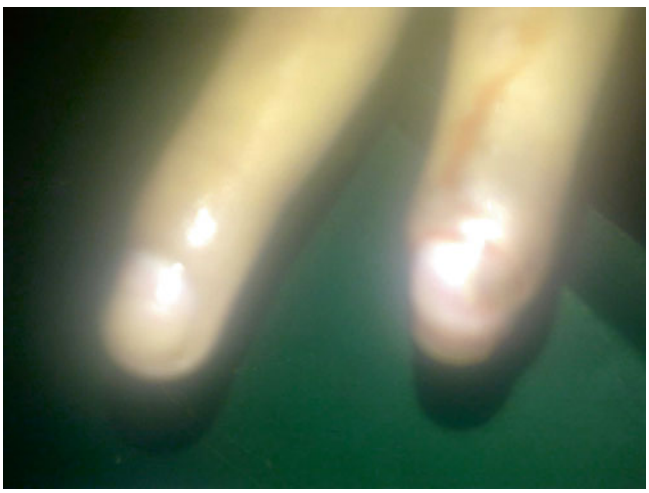
Photograph 23.8 Retrieved injured finger (crushed middle finger and its retrieval after 6 weeks)

Photograph 23.6 Retrieved injured finger (crushed thumb) after 6 weeks

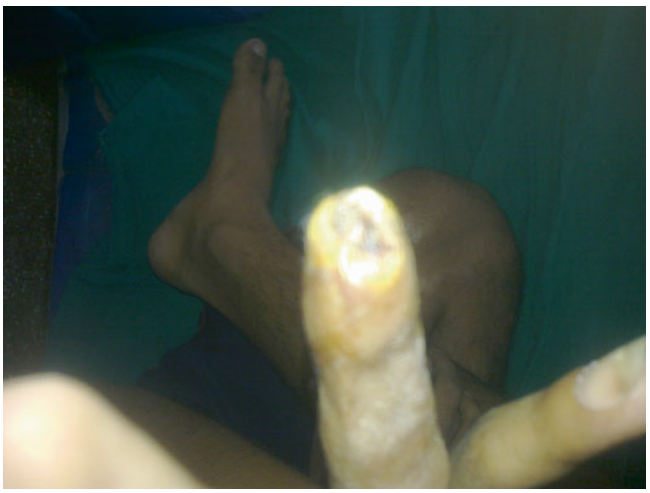
Photograph 23.9 Retrieved injured finger (crushed index finger and its retrieval after 6 weeks)



Photograph 23.7 Retrieved injured finger (crushed thumb and its comparison with the normal thumb) after 6 weeks



Photograph 23.10 Retrieved injured finger (crushed index finger and its retrieval after 6 weeks)



Photograph 23.11 A typical cut injury of the middle finger with a sharp knife. It would be now placed in a fatty tunnel in the abdomen. After its preparation in the OT

Discussion

The old concept that the fatty tissue is avascular in nature has been seriously questioned by the recent progress in stem cell biology which has allowed researchers to investigate distinct mesenchymal stem cell populations in divergent mammalian tissues and organs such as tendon [10], periodontal ligament [11], and synovial membrane [12] and body/tissue fluids such as the synovial fluid [13]. Despite the identification of stem cells from various sources, taking those stem cells adaptable for regenerative medicine applications in adequate quantities at the right time is a challenge. In this respect, an emerging body of literature suggests that redundant adipose tissue serves as an abundant, accessible, and reliable source of mesenchymal stem cells that can be readily harvested with minimal risk to the patient. Rapidly accumulating evidence suggests that adipose tissue-derived stem cells, especially from white adipose tissue, possess a far wider property of self-renewal and multilineage differentiation capacity, thereby highlighting their importance and effectiveness in regenerative medicine [14, 15].

Adipose tissue is present physiologically in multiple locations in the human body and is responsible for multiple functions (mechanical, endocrine, thermoinsulatory, and energy supplying). For example, in the case of breast reconstruction, the injection of adipose tissue not only partially reconstructs the amputated breast but locally supports better healing and prevents formation of connective tissue scar between the skin and muscles. What is interesting is the fact that in each and every case of placement of the injured crushed finger in the subcutaneous fatty tunnel, there is invariable neovascularization at the ischaemic finger zone. We believe this regeneration is a stem cell-mediated phenomenon with active transdifferentiation capacity on the basis of niche. Further study is in progress. Human adipose-derived stem cells (ASC) have all the positive potential for clinical applications. The use of ASC to provide adipose tissue for the reconstruction of soft tissue defects is well known. The clinical efficacy of ASC in soft tissue regeneration or reconstruction is accepted globally. Fat injection (FI), where the whole of the aspirated fat is reinjected without any processing to separate or concentrate the ASC, has been performed since 1893 for reconstructive purposes.

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Understanding the Science Behind Regeneration for Its Implications in the Medicine of the Future

24

Niranjan Bhattacharya and Phillip G. Stubblefield

The use of microbial and human cells for therapy is a new concept in medicine and has the potential to engineer a revolution in the pharmaceutical industry, which was earlier dominated by small-molecule drugs and was later revolutionized by the advent of biologics. This so-called "third pillar" of therapeutics will however depend on the development of the science of cellular engineering [1]. This science should provide a systematic framework for safely and predictably altering and regulating cellular behavior. The application of dynamic cell behavior in clinical research, based on the principle of replacement or repair of dead or damaged cells, will initiate the use of cell therapy in clinical medicine. This, in other words, is what consists regenerative medicine, which can also be labeled futuristic medicine. There is tremendous progress on key issues and on the understanding of the process of regeneration that are common to natural regenerative events. The crux of the problem is to determine or predict the regenerative capacity of a cell or group of cells including an organ and its effective functioning. The roles of stem cells, its participation or nonparticipation at the regeneration stimulus, which again depends on its ambience or niche; its capacity for transdifferentiation or dedifferentiation; as well as its cytokine support signals, are very crucial. These factors control the initiation and targeting of regenerative activity including its participation, proliferation, and patterning [2].

Regeneration activity is actually a natural activity. It has long been observed that salamanders and other creatures are able to regenerate a lost limb, a feature of ongoing development that is sadly lost to human beings. It may be remembered that way back, humans had tails, but since they got in the way when walking upright, nature shortened them until just the coccyx remains.

However, some residual activity (?) may remain. For example, a group of investigators examined the effect of human mesenchymal stem cell (MSC) implantation on the restoration of degenerative intervertebral discs (IVDs) in the rat coccygeal region in a unique rat coccygeal trauma model. Post-injury radiologic and histologic evaluations were performed at 2, 4, 6, and 8 weeks. Results suggested that MSC-injected segments (TS) retained disc height and signal intensity, but injured non-injected segments (IS) progressively lost disc height. Pathological results revealed that the TS group showed relative restoration of the inner annulus structure; however, the IS group showed destruction of the inner annulus structure [3].

This suggests the use of the plasticity of differentiated cells, the retention in regenerative cells with the maintenance of local cues such as positional identity. Nature's limb regeneration appears to always proceed in three stages: (1) initial wound closure, (2) generation of a blastema (a mass of undifferentiated or redifferentiated cells at the amputation site), and (3) differentiation or redifferentiation of cells to re-form the missing structures. The local formation of a blastema is very important because it is actually a growth zone of mesenchymal stem cells on the stump. The blastema can regenerate autonomously as a self-organizing system over variable linear dimensions. Brockes and Kumar reviewed what is known about amphibian limb regeneration and speculate on how these observations could inform application of stem cell and regenerative medicine to mammalian cases [4].

Regeneration of body parts has been observed in zebra fish as well. These fish regenerate their fins after trauma or injury. Here too, regeneration occurs through initial formation of a clump of undifferentiated cells, the blastema, which through growth and differentiation elaborates a replacement fin. This process is known as facultative regeneration by which an amputated or otherwise injured organ is re-formed.

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Homeostatic regeneration is the process that maintains a tissue under normal conditions. The organ's capacity to regenerate following amputation is positively correlated with the amount of cell turnover in that organ, which suggests that the processes share some molecular components. Wills et al. [5] tested whether the requirement for fibroblast growth factor (*FGF*) signaling in facultative regeneration also extended to homeostasis in zebra fish fins. When they reduced FGF signaling by daily expression of a dominant negative form of FGF receptor 1 from a heat shock-inducible promoter, the fish showed a progressive loss of distal fin tissue, having shortened fins with ragged edges after 60 days. When the heat shock regimen was abandoned, the fish reestablished normal fin morphology within 30 days. Similar fin atrophy occurred in animals lacking fgf20a, a FGF ligand specifically required for facultative regeneration.

Whitehead et al. [6] and Antebi [7] have identified one of the signaling factors critical to the formation of the blastema. In zebra fish, the *dob* mutation affects a gene that encodes signaling factor FGF20, which seems to be used specifically for regeneration rather than for normal embryonic development.

As mentioned earlier, under normal circumstances, in mammals too, the process of regeneration of limbs or digits occurs in a slightly modified form in three usual stages: wound closure, generation of a blastema, and differentiation or redifferentiation of cells to re-form the missing structures [6].

In neonatal and adult mice, regeneration of the digit tip occurs by a mechanism distinct from that by which the digit is generated in an embryo. Digits amputated proximal to the middle of the third phalangeal (P3) element do not regenerate, but digits amputated distal to the middle of P3 regenerate through a process of intramembranous ossification, in which bone is formed without a cartilage intermediate [7].

Again, the process is different in the case of limb and digit formation in the embryo which occurs through a process of endochondral ossification, in which bone formation proceeds through a cartilage intermediate. Yu et al. [8] report that treatment with bone morphogenetic protein 2 (BMP2) or BMP7 conferred regenerative capacity to digits amputated proximal to the middle of P3. BMPs were required for normal digit regeneration, because application of the BMP inhibitor Noggin to digits amputated distal to P3 prevented regeneration, and genes encoding BMP2 and BMP7 were upregulated in the blastemas of amputated digits as compared to unoperated digits and to the portions of operated digits proximal to the amputation site.

It has also been shown that application of beads coated with BMP2 or BMP7, but not BMP4, induced regeneration of digits amputated proximal to the middle of P3, and regeneration continued even after removal of the beads. Furthermore, digits amputated proximal to the middle of P3 and treated with BMP7 regenerated by a mechanism fundamentally different from that followed by digits regenerated without treatment after amputation distal to the middle of P3.

The skeletal elements of digits amputated distal to P3 and left untreated proceeded through direct membranous ossification, whereas digits amputated proximal to P3 and treated with BMPs proceeded through endochondral ossification, as indicated by the expression of endochondral marker genes. Thus, it may be concluded that exogenous application of BMPs can induce redeployment of the embryonic developmental limb program at an amputation site, a process which is distinct from the adult type of regeneration normally observed in untreated amputated digits, indicating that it may be possible to induce regeneration in tissues not normally competent to regenerate [8]. There is another signaling pathway known as the Wnt pathway which enables *Xenopus* tadpoles to regenerate their tails [9].

Various genes involved in the production of reactive oxygen species (ROS) are expressed during regeneration in a complex biochemical interaction. Love et al. found that H_2O_2 was required for tail regeneration and induced a signaling cascade that was separate from pathways required for wound healing. Analysis with either a fluorescence reporter for H_2O_2 or H_2O_2-sensitive fluorogenic dyes in vivo revealed an increase in intracellular H_2O_2 concentration that peaked within 1 h of amputation and remained high during tail regeneration. H_2O_2 production preceded the recruitment of inflammatory cells to the site of injury.

Morphant tadpoles deficient in SPIB (a lymphoid cell-specific transcription factor) had diminished numbers of inflammatory cells in the regenerative bud tissue but similar intracellular concentrations of H_2O_2 to controls, indicating that amputation-induced ROS was produced by noninflammatory cells. Treatment with either diphenyleneiodonium (DPI) or apocynin (APO) – chemicals that inhibit the NADPH oxidase (NOX) complex – reduced ROS concentration, impaired tail regeneration, and decreased the number of proliferating cells to a greater extent than did treatment with the antioxidant MCI-186 (MCI).

Removal of MCI, but not DPI or APO, restored tail regeneration. Furthermore, amputation-induced Wnt–β-catenin signaling and expression of a downstream transcriptional target FGF20 were reduced after amputation in DPI-, APO-, or MCI-treated tadpoles. Morphant tadpoles deficient in the NOX complex subunit CYBA (cytochrome b-245 α polypeptide) showed defects in H_2O_2 production and tail regeneration similar to those in DPI- or APO-treated tadpoles. Morphant tadpoles deficient in FGF20 could heal the wound at the amputation site but failed to fully regenerate tails and showed specific defects in axial tissue regeneration. Together, the findings indicate that NOX complex-induced ROS signaling is critical to initiate regeneration and facilitates signaling through the Wnt–β-catenin–FGF20 pathway [9].

Most metazoans have at least some ability to regenerate damaged cells and tissues, although the regenerative capacity of an individual varies depending on the species, organ, or developmental stage [10, 11]. Translating these minor examples may help us to understand the mysteries of complex neuro-regeneration in the human system.

The static and dynamic cell behavior in clinical research is practically the initiation of cell therapy in clinical medicine with its integral principle of replacement or repair of dead or damaged cells with everlasting regenerative cells or the stem cells. The possibilities of futuristic medicine including an understanding of the promise of facultative regeneration, will unfold in the coming years, providing hopes for clinical therapy for regeneration of damaged tissue including limbs.

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25 Uterine Synechia: A Preliminary Communication on an Attempted Treatment of the Condition with Intrauterine Instillation of Autologous Bone Marrow Mononuclear Cells

Niranjan Bhattacharya, Sushanta Kumar Banerjee, and Dhruba Malakar

Introduction

The uterine cavity is lined by the endometrium. This lining is composed of two layers, the functional layer (adjacent to the uterine cavity) which is shed during menstruation and an underlying basal layer (adjacent to the myometrium) which is necessary for regenerating the functional layer. Thorough curettage and removal/partial removal of the basal layer often leads to a condition known as synechia, or Asherman's syndrome (AS) or Fritsch syndrome, where there is intrauterine adhesion of the walls of the uterus, as a result of which the estrogen-sensitive areas are lost leading to a condition where patients experience secondary menstrual irregularities characterized by a decrease in flow and duration of bleeding (amenorrhea, hypomenorrhea, or oligomenorrhea) [1].

A forceful or thorough dilation and curettage (D&C) performed after a miscarriage or delivery or for medical abortion can lead to the development of intrauterine scars resulting in adhesions that can obliterate the cavity to varying degrees. In the extreme, the whole cavity can be scarred and occluded. Depending on the degree of severity, synechia may result in infertility, repeated miscarriages, pain from trapped blood, and future obstetric complications [2]. If the problem is left untreated, there is evidence that the obstruction of menstrual flow resulting from adhesions can lead to endometriosis [3].

Uterine synechia can also result from other pelvic surgeries including cesarean sections and myomectomy, neglected IUDs, pelvic irradiation, schistosomiasis [4], and genital tuberculosis, which is difficult to treat [5]. Other causes include septate uteri and other Müllerian defects; patients suffering from these problems are at a higher risk of sustaining endometrial damage after blind D&C due to their atypical anatomical presentation. An artificial form of AS can be surgically induced by endometrial ablation in women with excessive uterine bleeding. The gold standard of diagnosis is through hysteroscopy. Imaging by sonohysterography or hysterosalpingography is the best method for examining the extent of scar formation.

Existing Basic Treatment Options

Operative adhesiolysis is the standard procedure after which the inside of the uterus is visualized properly. Microscissors are usually used to cut adhesions. In more severe cases, adjunctive measures such as laparoscopy are used in conjunction with hysteroscopy as a protective measure against uterine perforation. Methods to prevent further adhesion reformation include the use of mechanical barriers like the Foley catheter, saline-filled Cook medical balloon uterine stent, or an IUD and gel barriers to keep opposing walls apart during healing. Antibiotic prophylaxis is often necessary. A common pharmacological method for preventing reformation of adhesions is sequential hormone therapy with estrogen followed by a progestin to stimulate endometrial growth and prevent opposing walls from fusing together.

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Patients who remain pregnant after treatment of intrauterine adhesion (IUA) may have an increased risk of having abnormal placentation including placenta accreta [6] where the placenta invades the uterus more deeply, leading to complications in placental separation after delivery. Premature delivery [7], second-trimester pregnancy loss [8], and uterine rupture [9] are other reported complications.

Suggested Alternative Treatment

This paper presents an alternative treatment of synechia/AS with the use of autologous bone marrow mononuclear cells, which were instilled into the uterine cavity of five patients in an attempt at regeneration of the endometrium with the hypothetical objective of restoring normal menstruation and general normalcy of the uterine cavity due to possible transdifferentiation of viable autologous stem cell component of the bone marrow which is injected with mononuclear component niche provided from their own bone marrow.

Materials and Methods

Five patients who were suffering from uterine synechia and varying degree of amenorrhea were enrolled for the present study at Bijoygarh State General Hospital (from 2002 to 2006) to see the effect of autologous bone marrow mononuclear cell instillation inside the uterus aseptically in the OT and to see its effect on the restoration of the menstruation.

They were suffering from persistent secondary amenorrhea to oligo-/hypomenorrhea for 7–18 months and did not respond with conventional treatment of estrogen + progesterone cyclic supplementation. Earlier before enlistment, there was correction of thyroid status and nutrition of the patient. In all cases the diagnosis is based on hysteroscopy and other supportive blood examination to exclude other chronic diseases. For confirmation of tuberculosis, we did DNA (PCR) koch's examination of the endometrium. Synechia in four cases was due to excessive curettage, and in the remaining 1 case, tuberculosis was affecting the endometrium (PCR confirmed).

They were taken as subjects for the present study protocol after passing through the institute-based ethical committee and special consent protocol including medicolegal consent (affidavit from court). We lost follow-up of one case after her inclusion in this protocol.

Twenty-five to forty milliliters bone marrow was collected from the patient as per standard protocol of antiseptic and aseptic precaution and 2 % Xylocaine infiltration from the site of collection, i.e., the iliac crest bone. After a collection of bone marrow aspirate, mononuclear cells were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) and the erythrocytes were lysed with H_2O cultivation, 1×10^6 BMCs/mL was placed in Teflon bags (VueLife, CellGenix) and cultivated in X-VIVO 15 Medium (BioWhittaker) supplemented with 2 % heat-inactivated autologous plasma. The next day, BMCs were harvested and washed three times with heparinized saline before final resuspension in heparinized saline. Viability was tested to be 96.3 %. This is now prepared for injection in the OT through the transcervical route very slowly with the head end of the patient slightly lowered.

Case History

Table 25.1

Collection

In all cases autologous bone marrow mononuclear cells were collected, and 10 cc of the mononuclear cell (1×10^6 BMCs/mL) was injected through the uterine route slowly (after examination of the constituent fractions and after confirmation of the nonexistence of any preexisting diseases).

Table 25.1 Clinical diagnostic and therapeutic features of uterine synechia

Name, age, parity, and serial no.	Problem	Diagnosis by hysteroscopy	Surgery	Bone marrow instillation with objective of restoration of normal menstruation or improvement of flow
Serial no. 1: Mrs. K. B., 29 years	Amenorrhea	Uterine synechia post curettage	Adhesiolysis	10 cc bone marrow mononuclear cell instilled inside the uterus. Restoration of normal menstruation after 3 months was noted
Serial no. 2: Mrs. G. B., 24 years	Oligomenorrhea	Uterine synechia post curettage	Adhesiolysis	10 cc bone marrow instilled inside the uterus. Definite improvement was noted of menstrual flow after 4 months
Serial no. 3: Mrs. D. B., 34 years	Oligomenorrhea	Uterine synechia post curettage	Adhesiolysis	10 cc bone marrow instilled inside the uterus. Definite improvement was noted of menstrual flow after 5 months
Serial no. 4: Mrs. G. S., 25 years	Hypomenorrhea	Uterine synechia with background tuberculosis	Adhesiolysis	10 cc bone marrow instilled inside the uterus. Definite improvement was noted of menstrual flow after 6 months
Serial no. 5: Mrs. K. B., 35 years	Hypomenorrhea	Uterine synechia with background tuberculosis	Adhesiolysis	10 cc bone marrow instilled inside the uterus. Definite improvement was noted of menstrual flow after 6 months

Discussion

Classically, Asherman's syndrome is defined as the complete obliteration of the uterine cavity with adhesions resulting in the core symptoms of amenorrhea and infertility [10]. Another condition known as intrauterine adhesion refers to the partial adherence of the endometrial surfaces with fibrotic tissue, and the clinical presentation depends on the stage and grade of involvement of the endometrium with the age and background of the medical disease or the hormonal condition of the patient. After hysteroscopic confirmation of the lesion, adhesiolysis, IUD insertion, and cyclic estrogen + progesterone support are generally the standard practice. However, this treatment may not be enough if there is no response with hormonal supplementation even in high dosage.

It may be noted here that in contrast to other damaged mucosa like the intestine where scarring is a rule, the endometrium behaves differently where, following menstruation or parturition, the endometrial surface epithelium repairs without scarring [11].

The main question this paper tries to address is whether using autologous bone marrow-derived stem cell-rich mononuclear cell populations, which actually regenerated the endometrium in the five cases mentioned in Table 25.1, was also able to sufficiently help to restore menstruation. It is well known that adult bone marrow, like many other organs, harbors small adult stem cell populations. Adult stem cells are rare undifferentiated cells that have been identified by their functional properties in most adult tissues and organs in the body. Their role is to maintain tissue homeostasis, providing replacement cells lost through cellular turnover and following tissue damage. HLA- and gender-mismatched transplant studies in human and mouse suggest that bone marrow-derived cells may incorporate into the endometrium in a setting of ongoing tissue damage and inflammation [12]. The actual bone marrow cell type that transdifferentiates into endometrial cells has not yet been identified and could be myeloid cells, hemopoietic stem cells, MSC, or endothelial progenitor cells. A recent report noted that rare populations of epithelial progenitor cells and mesenchymal stem/stromal cells (MSC) were identified in the human endometrium [13]. Functional approaches have been used to identify candidates for endometrial, epithelial and stromal stem/progenitor cells, due to lack of known specific endometrial stem cell markers. Endometrial mesenchymal stemlike cells (eMSC) have prospectively been isolated recently as CD146(+)PDGF-Rβ(+) cells and have been found in both basalis and functionalis as perivascular cells. Epithelial progenitor cells have been detected in colony-forming unit assays but their identity awaits elucidation [14].

Conclusion

Transdifferentiation of the stem cell is one of the fundamental properties of stem cells. The present preliminary study reconfirmed that stem cells, irrespective of the source of origin, become converted to specific cells on the basis of guidance provided by the niche.

All the five patients completed the trial of mononuclear cell collection from bone marrow and instillation inside the uterus through the transcervical route. All the patients definitely had improvement in their menstrual bleeding; and in one case of complete amenorrhea, there was resumption of near-normal menstrual flow. Though transdifferentiation of the stem cells in the niche of the endometrium is the simple explanation, further chemo-cytokine support and hormonal coordination are to be studied in detail to learn about the true molecular background which can explain why there is resumption of period in 3 months in one case and 4/5/6 months in other cases.

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26 Freshly Collected Amniotic Fluid and Amniotic Membrane as Dressing Material for Leprosy Patients with Gangrene: A Preliminary Report on an Experience with Six Cases

Niranjan Bhattacharya, Pratap N. Gupta, and Dhruba Malakar

Introduction

Leprosy, which was known to ancient India as "Kushta Roga," is thought to have originated from India. India is now having the maximum numbers of leprosy cases and accounts for 67 % of the total prevalence and 73 % of total new cases detected worldwide. Huge numbers of new cases have been detected in recent years (in 2013, new cases detected were 782,501) because of the adoption of a new strategy, the Modified Leprosy Elimination Campaign (MLEC), and an effective health education campaign. Presently, 70 % of the total new caseloads of India are from five most heavily infected states. There is a significant improvement in the overall situation as is evident from the steady decline of the prevalence rate from 38.6 cases per 10,000 population in 1985 to 5.0 per 10,000 population in 1999 [1–3].

Leprosy still remains an important public health problem for many parts of the world. Gangrene associated with leprosy is not rare and can have a number of causative mechanisms.

The detailed demographic profiles and clinical findings were noted from the predesigned clinical proforma. A slit-skin smear for acid-fast bacilli (AFB) was done in all cases from the area of sensory loss. A skin biopsy was done from the area of sensory impairment to study histopathological changes. Further investigations such as nerve conduction velocity studies (NCV), fine-needle aspiration cytology (FNAC), and nerve biopsy (superficial nerve twigs) were done if indicated in patients whenever there was difficulty in clinical diagnosis. PNL (polyneuritic leprosy) is a distinct subset of disease frequently seen in India. There is a need to pay more attention to this form of leprosy and diagnose and treat patients earlier to prevent deformities and sequelae of nerve damage [1]. The treatment for PB (paucibacillary) leprosy continues up to 6 months, but in MB (multibacillary) leprosy with a high bacterial index, a longer duration of multi-drug therapy (MDT) may be required. Following completion of MDT, many cases with deformity accumulate and their care forms a neglected part of many control programs. In addition to strengthening the infrastructure, simple techniques must be imparted to those with deformities and disabilities. From the leprosy clinic affiliated to Bijoygarh State General Hospital, Jadavpur, Calcutta 32, India, we treated five cases of leprosy gangrene (Figs. 26.1, 26.2, and 26.4 show typical gangrene of the great toe associated with leprosy and infestation with maggots) and one case of leprosy with autoamputation (Fig. 26.3) of toe digits, which showed

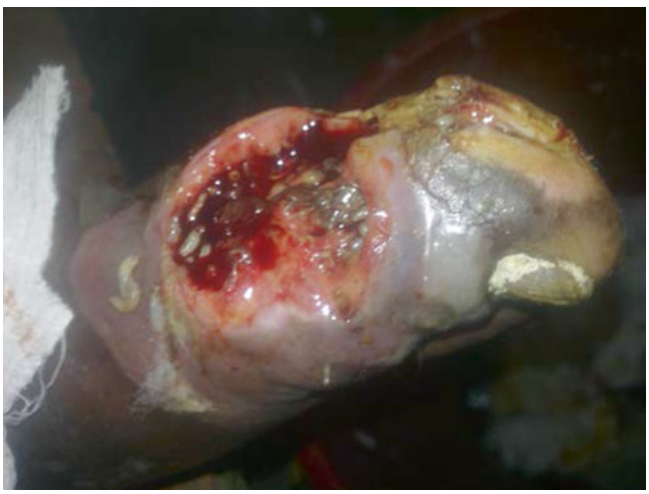

Fig. 26.1 Showed typical gangrene of the great toe associated with leprosy, infested with maggots

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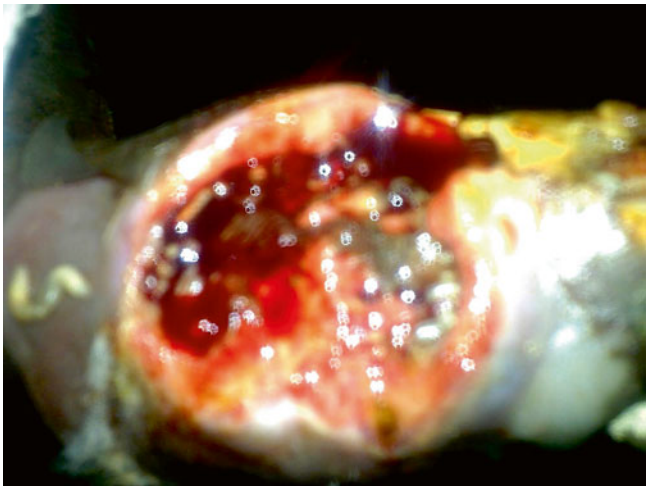

Fig. 26.2 Showed typical gangrene of the great toe associated with leprosy, infested with maggots (at a different angle)

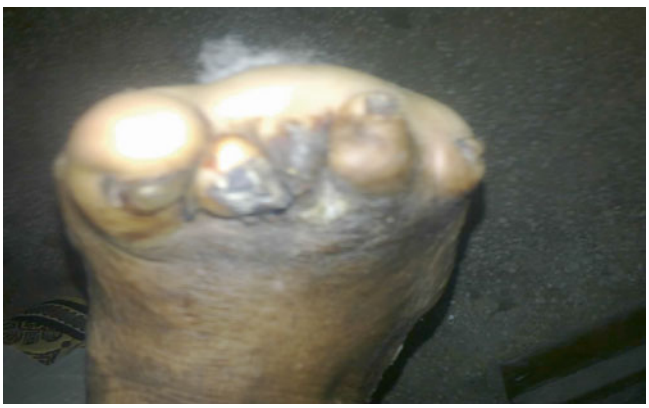

Fig. 26.3 Showed typical autoamputation of the toes associated with leprosy

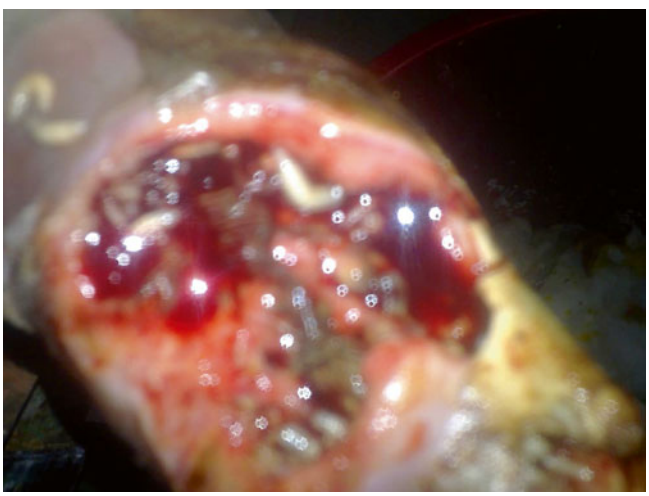

Fig. 26.4 Showed typical gangrene of the great toe associated with leprosy, infested with maggots (from another angle)

clinical improvement with the application of freshly collected amniotic membrane and amniotic fluid after its proper screening for HIV (1 and 2) and also hepatitis B, C, etc.

Result and Analysis
(Summarized in Table 26.1)

Six cases of leprosy were recruited for this present study of gangrene in the background of leprosy. All the cases were treated with (1) removal of maggots, with normal saline for initial removal of dirt and debris, followed by (2) sprinkling of copious amounts of freshly collected clear amniotic fluid at the site of the leprosy wound (after 5–10 min), and, lastly, with the application of amniotic membrane (3) at the wound site (amniotic or the fetal side of the amniotic membrane in case of superficial or partial thickness skin wound for early epithelialization and maternal attachment site or the chorionic site in deep wound to improve circulation through angiogenesis-supporting cytokine content of the chorionic site of the membrane).

This amniotic membrane was kept in the amniotic fluid, which was freshly collected from consenting donor mothers who were negative for Venereal Disease Research Laboratory test for syphilis (VDRL), hepatitis B and C, and HIV (1 and 2) and had undergone cesarean section. The entire operation was done in the OPD and it may be noted that this is an absolutely painless process. In all cases within 3 months, there is healing by granulation tissue followed by epithelialization. However, follow-up was continued till 6 months to check if there was any reappearance of the leprosy ulceration. All patients took standard antileprosy treatment meticulously.

Discussion

Wound healing is a very complex process that is tightly regulated to achieve wound repair. The process has three important components, i.e., inflammation, proliferation, and maturation. Following the initial tissue injury, inflammatory mediators known as cytokines are released from the injured tissue cells and wound blood clots, after which the inflammatory phase initiates. Then, the proliferation stage begins several days after injury. In this stage, platelet degranulation activates the coagulation cascade, and the resultant fibrin clot serves as a scaffold for the proliferation phase of wound healing. During the proliferative phase, fibroblasts in the extracellular matrix increase and synthesize the tissue components, such as proteoglycans, fibronectin, and collagen. New vessels and epithelium are formed as rapidly as possible to maximize the tissue replacement dynamics. All wound cells are maximally active and are sensitive to factors that regulate cell proliferation and protein biosynthesis.

Table 26.1 Result and analysis

Serial no. and name	Age	Sex	Background disease	Clinical presentation: maculopapular anesthetic lesion with adjacent nerve thickening. Patient reporting with gangrene or autoamputation of the leprotic lesion	Amniotic fluid + amniotic membrane dressing (placed at the gangrene site after proper antiseptic dressing procedure)	Follow-up visit monthly for 6 months or till there is healing
[1] PS	35	F	Leprosy with gangrene or autoamputation	do	do	Satisfactory
[2] VD	42	F	do	do	do	Satisfactory
[3] SD	34	F	do	do	do	Satisfactory
[4] SM	38	F	do	do	do	Satisfactory
[5] KR	33	F	do	do	do	Satisfactory
[6] JM	38	F	do	do	do	Satisfactory

In order to augment the complex process of wound healing, investigators have used amniotic membrane as a temporary dressing material with the belief that it helps in proliferation and repair. The balance between tissue degradation and biosynthesis permits remodeling of the provisional tissue and its ultimate repair. In order to augment the complex process of wound healing, application of freshly collected and screened amniotic membrane, which have a poor expression of HLA-A, -B, -C, -DR antigens, or beta-2 macroglobins on their surfaces, have proven to be extremely effective [3]. However, there has been some recent controversy regarding this topic. It has been noted that amniotic membrane epithelial cells display some degree of antigenicity and immunogenicity as allografts due to the presence of some (though definitely less than adult) Major Histocompatibility Complex (MHC) class I and II antigens.

On the other hand, viable human amniotic epithelial cells (HAECs) have been shown to elicit beneficial effects on secretion of anti-inflammatory factors. It has been seen that topical application of culture supernatant from HAECs leads to profound suppression of suture-induced neovascularization in the cornea. In addition interleukin (IL)-1 beta mRNA was suppressed in a cauterized cornea where the amniotic membrane was applied. These results suggest that amniotic cells are a source of soluble anti-inflammatory factors that suppress inflammation [4]. In spite of the scientific controversy, the clinical and practical impression suggests that this dressing is extremely effective. It speeds up the healing process and reduces pain. The relative ease of procurement and preparation and its low cost and easy availability project the amniotic membrane as an ideal temporary skin substitute. The amniotic membrane has been utilized in various studies to cover the burn wound dressing for less exudation of protein and electrolyte, as well as for its bio-friendly nature and hypoantigenic qualities apart from the cytokine support it lends to wound healing. Pregnancy-specific biological substance, i.e., amniotic fluid, also plays a very important role if used in other injuries. It has to be remembered in this connection that nature washes the vaginal canal of the mother with the amniotic fluid before the birth of a baby in all species in order to prevent infection to the baby. This is nature's proof of the sterile and bactericidal properties of the amniotic fluid. In addition, the amniotic fluid possibly possesses a lubricating effect due to its higher viscosity and protein and cellular composition and may also have a reparative effect due to the progenitor cell/stem cell component in it, i.e., the epithelial and the mesenchymal stem cell population. The stem cells of the amniotic fluid are capable of differentiating into multiple lineages; this may be valuable for therapy. In this context it is relevant to mention that a group of renowned investigators have reported in *Nature Biotechnology* on the isolation of human and rodent amniotic fluid-derived stem (AFS) cells that express embryonic and adult stem cell markers. Undifferentiated AFS cells expand extensively without feeders, double in 36 h, and are not tumorigenic. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells were noted to be broadly multipotent [5]. Use of amniotic membrane for covering the wound decreases oozing from the wound site after debridement and thus decreases the need for blood and albumin transfusion and causes less electrolyte imbalance. Amniotic membrane also possesses some antibacterial characteristics, namely, bacteriostatic effect on gram-positive bacteria due to the lysozyme content.

The amniotic membrane also helps in the epithelialization of the wound. An important clinical scientist from China, Prof. Andrew Burd of the Chinese University of Hong Kong, himself a renowned plastic and reconstructive surgeon, has calculated the global production of placentas, amniotic fluid, and the amniotic membrane which are noted in his article. This will convey a picture of the massive global wastage of such materials when we throw them into the dustbin for eventual destruction through incinerators. Before analyzing the utilization potential of pregnancy-specific biological substances in wound, it is necessary to give a brief account of the substance compositions and properties and the contemporary scientific advances in the field of stem cell research which relate to these important substances.

Amniotic Epithelial Cells

Amniotic epithelial cells are isolated following the stripping of amniotic membrane from the chorion by trypsin digestion [6]. Such procedure allows for selection of relatively homogeneous cell suspension, which attach to plastic in in vitro culture. Contrary to mesenchymal stromal cells (MSC-type cells), amniotic epithelial cells need the addition of epidermal growth factor (EGF) to Dulbecco's Modified Eagle's Medium (DMEM) supplemented with forward scatter (FSC).

Cells grow throughout two to six passages, displaying typical epithelial morphology. The expression of both CD90 antigen and HLA-A, HLA-B, and HLA-C (human leukocyte antigens) increases in culture – the initial expression levels are too low for using these antigens as identification/selection markers for freshly isolated epithelial cells. Among the other markers, cells express molecular markers of pluripotent stem cells (SRY-related HMG-box gene SOX-2, octamer-binding protein 4 Oct-4, and Nanog). Contrary to placental MSCs, epithelial cells do not express the Cd49d marker. Both the molecular markers and differentiation experiments suggest that amniotic epithelial cells are pluripotent, having not only adipogenic [7], osteogenic [8], and chondrogenic potentials but also myogenic, cardiomyogenic, neurogenic [9], pancreatic [6], and hepatogenic [10] potentials.

Mesenchymal Stromal Cells from Amniotic and Chorionic Regions

Amniotic MSCs are isolated from amnions at any gestation stage when the placenta is fully developed. The amnion must be carefully dissected from fetal membranes to avoid the presence of maternal cells. The most popular isolation protocol is based on the two-step digestion procedure, first with trypsin and subsequently amniotic epithelial cells from the amniotic epithelial region or amniotic mesenchymal stromal cells (MSC) from the amniotic mesenchymal region, to name a few [11]. Of these cells, amniotic and chorionic mesenchymal stromal cells represent characteristics similar to in vitro growth characteristics and exhibit surface antigen expression and differentiation potential. Both cell types are hematopoietic markers – negative for (CD34–, CD45–), HLA-DR–, and positive for markers attributed for MSC: CD73, CD90, and CD105 [12]. The characteristics of the amniotic epithelial cells are somewhat more complex – they are able to proliferate shorter than MSCs in in vitro culture, proliferate only in higher densities in the presence of epidermal growth factor (EGF), and change the expression of selected markers (HLA-A, HLA-B, HLA-C, CD90) depending on the culture time. This latter phenomenon may suggest that the amniotic epithelial cells are a heterogeneous population being subsequently selected to higher homogeneity by culture conditions [13].

Conclusion

Introduction of MDT into the National Leprosy Eradication Program (NLEP) of India has brought a decline in both the burden of the disease and the detection of new cases in the country. Despite this success, MDT has had many problems like remarkable relapse rate, nonadherence to the MDT, and the emergence of drug resistance associated with it. Moreover, there is no new MDT regimen at present, which could solve all these problems. The current situation suggests that we should look for alternative solutions in the delivery of leprosy-related services. With the introduction of Accredited Social Health Activists under the National Rural Health Mission, there is an opportunity to control some of these problems associated with MDT. This involves the artful and innovative cooperation of the health worker, patient, and the community [2]. In the total socioeconomic perspective of India, beggars use leprosy and the ulceration often associated with festering to solicit sympathy and support.

To many of our scientific colleagues, pregnancy is an inflammation. The stem cells which are pregnancy specific are embryonic stem cells. These totipotent cells progressively differentiate and eventually become pluripotent in its characteristics after differentiation. These cells are associated with some materials which appear during pregnancy and disappear after the pregnancy or become a biological waste. Examples are the placenta, amniotic membrane, and the amniotic fluid. They nourish the pregnancy with specific and nonspecific cells with stem cell property or even cells with partial stemness till birth. This process is a complex process of differentiation or even dedifferentiation. All the clinical utilities of these pregnancy-specific biological substances are under critical scientific review for its extreme utility in clinical medicine. The present study is a phase 1 study to show the efficacy and visibility of epithelial and mesenchymal stem cell-rich amniotic membrane and amniotic fluid application in cases of nonhealing neuropathic ulcer of leprosy.

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Use of Fetal Skin and Amniotic Fluid Dressing for Non-healing Burn Patients in Pediatric Age Group: A Study of 5 Cases

27

Niranjan Bhattacharya, Sushanta Kumar Banerjee, and Dhruba Malakar

Introduction

Autologous partial- or complete-thickness skin grafting is the gold standard for treatment of deep second- and third-degree burns. Available bioengineered skin products also necessitate this two-step surgical procedure. Investigators also developed fetal skin constructs, on native horse collagen, to improve healing of such degree burns [1]. Judith Hohlfeld et al. claimed that fetal skin cells might have great potential to treat burns and eventually acute and chronic wounds of other types. The same investigators suggested that engineering of fetal tissue has a high potential for the treatment of acute and chronic wounds of the skin in humans as these cells have high expansion capacity under simple culture conditions and one organ donation can produce master cell banks which can fabricate over 900 million biological bandages (9 × 12 cm). In a phase 1 clinical safety study, cases are presented for the treatment of therapy-resistant leg ulcers. All eight patients, representing 13 ulcers, tolerated multiple treatments with fetal biological bandages showing no negative secondary effects and repair processes similar to that seen in 3rd-degree burns [2].

Fire-related deaths are a neglected public health issue in India. These deaths are mostly due to kitchen accidents, self-immolation, domestic violence, and even dowry-related abuses. Death associated with burn injuries primarily depends on the age of the patient, the percentage of the body surface burned, and associated smoke-inhalation injury apart from the role of preexisting diseases such as diabetes, hypothyroidism, clinical and subclinical tuberculosis, and arthritis, nutrition level, etc. In a report published in *Lancet*, 163,000 fire-related deaths were reported in 2001 in India, and 106,000 of these were of women, mostly between 15 and 34 years of age. The average ratio of fire-related deaths of young women to young men was 3:1.1.

Wound sepsis and chronic wound formation are two frequent associates of burn injuries, which enhance the mortality and morbidity of burn patients. Chronic wounds are defined as wounds that have not proceeded through orderly and timely reparation to produce anatomic and functional integrity after 3 months. The ability of fetal skin wounds to heal without scar formation is remarkable. The mechanisms that endow the fetus with this unique healing ability remain unknown.

Material and Method

Fetal skin for donation and amniotic fluid for dressing are aseptically and ethically collected from informed consented mothers undergoing hysterotomy and ligation for family planning purposes approved by the state and central government guidelines. Each case was approved by the Institutional Ethical Committee of Bijoygarh State General Hospital, Jadavpore, Calcutta. They, i.e., both recipient and donor, completed the voluntary consent form before enlistment for the fetal skin transplant protocol. Both the donor and recipient had hepatitis B and C screening, HIV 1 and 2 screening, and others as per the transplant guidance protocol for the state and its upgradation from time to time.

Upon arrival, the burn patients (Figs. 27.1, 27.2, 27.3, 27.4, and 27.5), in the present series, were treated with initial normal saline wash to remove dirt and debris followed by cleaning the burnt area with freshly collected amniotic fluid aseptically collected and screened from cases of planned

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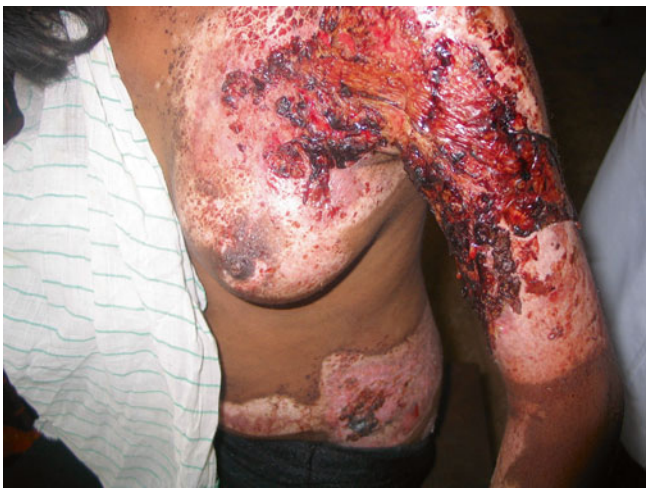

Fig. 27.1 Photograph of patient serial no. 2

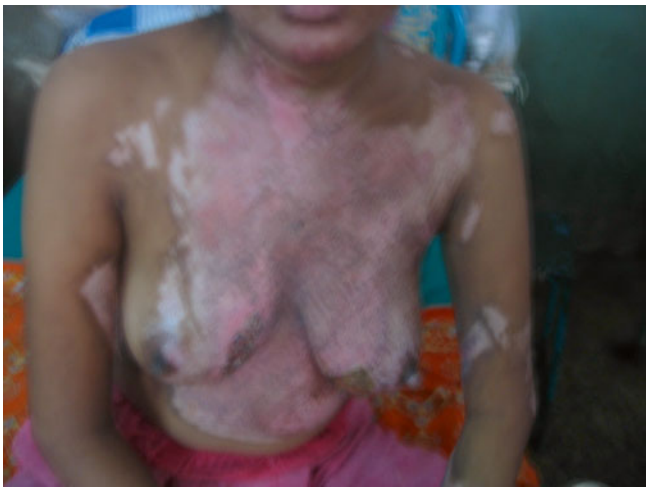

Fig. 27.4 Photograph of patient serial no. 4

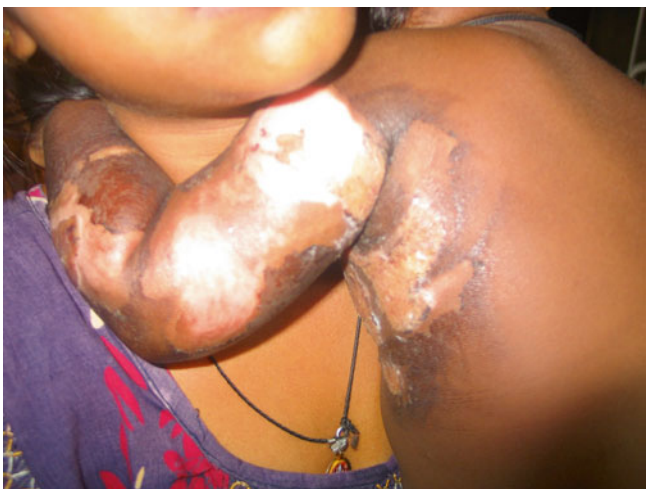

Fig. 27.2 Photograph of patient serial no. 1

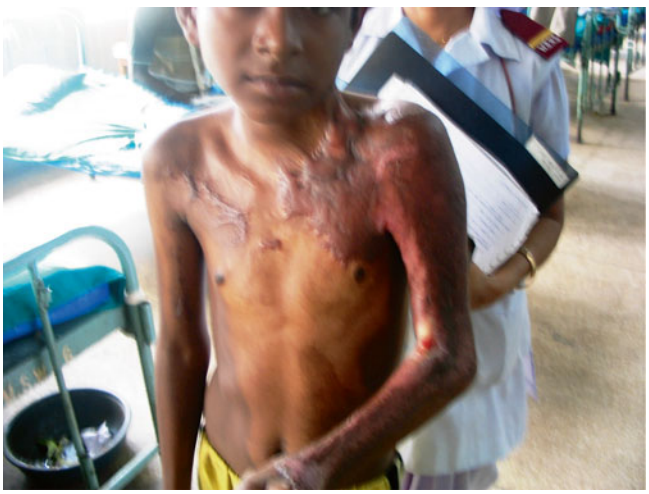

Fig. 27.5 Photograph of patient serial no. 5

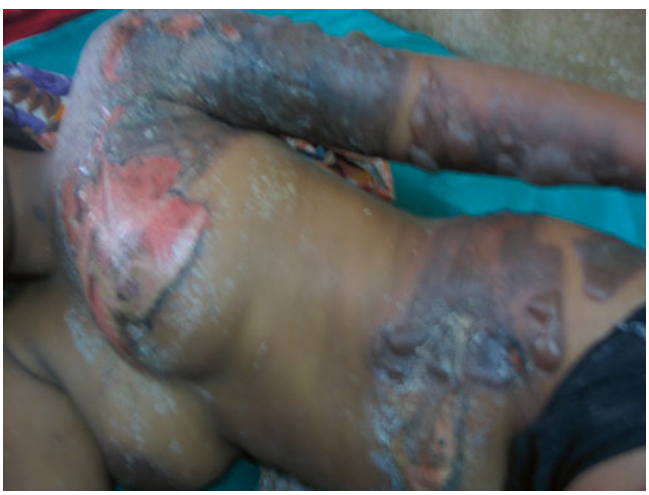

Fig. 27.3 Photograph of patient serial no. 3

cesarean section once to twice a week depending on availability. In cases of joints like elbow, knee, and other non-healed areas, freshly collected full-thickness fetal skin (below 20 weeks of gestation) is applied on the basis of availability. The fetal full-thickness skin is cut immediately in thin slices (1 mm) and placed on a fresh Sofra-Tulle gauze keeping a little gap, i.e., 3 mm, between two fetal skin constructs before its final placement over the selected site.

Result and Analysis
(Summarized in Table 27.1)

The developing fetus has the remarkable ability to heal dermal skin wounds by regenerating normal epidermis and dermis with restoration of the extracellular matrix architecture, strength, and function. The biology responsible for scarless

Table 27.1 Result and analysis

Serial no., age, sex	Clinical short profile	Conclusion
1. 2 years, M	15 % burn with dehydration and infection	Normal saline dressing followed by amniotic fluid spraying and then complete-thickness fetal skin (thin 1 mm long slices of fetal skin) was placed over Sofra-Tulle dressing and applied at the raw burnt area along with systemic antibiotics, pediatric fluid, vitamins, minerals, etc.
2. 18 years, F	25 % burn with dehydration and infection	Same principle
3. 17 years, F	18 % burn with dehydration and infection	Same principle
4. 17 years, F	14 % burn with dehydration and infection	Same principle
5. 11 years, M	25 % burn with dehydration and infection	Same principle

wound healing in skin is a paradigm for ideal tissue repair. This regenerative capacity is lost in late gestation when fetal wounds heal with fibrosis and scar. Early in gestation, fetal skin is developing at a rapid pace in a unique environment. Investigation of normal skin embryogenesis and comparison between early scarless and late scarring fetal wounds have revealed distinct differences in inflammatory response, cellular mediators, wound contraction, cytokines, growth factors, and extracellular matrix modulators [3]. In the present series, 5 pediatric patients (within 18 years of age) were treated with screened fetal skin collected from aborted fetuses (14–18 weeks old) donated by their respective mothers for medical research. The age of the pediatric patients varied from 8 to 17 years with their burned area varying from 14 to 25 %. All the patients responded favorably with fetal HLA randomized skin transplant. There was some residual scarring in two cases who reported to us with a chronic nonhealing ulcer due to burn treated with conventional methods outside our institution.

Discussion

Although there is a transition period between scarless and scar-forming repair, scarless healing also depends on wound size and the organ involved. The ability to heal scarlessly, furthermore, appears to be intrinsic to fetal skin. Unique characteristics of fetal fibroblasts, inflammatory cells, extracellular matrix, cytokine profile, and developmental gene regulation may be responsible for the scarless phenotype of early-gestation fetal wounds [4].

Wound healing in fetal skin is characterized by the absence of scar tissue formation, which may not be dependent on the intrauterine environment and amniotic fluid. Fetal cells have the capacity of extraordinary expansion, and some authors describe herein the development of a fetal skin cell bank where from one organ donation (2–4 cm^2), it is possible to produce several hundred million fetal skin constructs of 9×12 cm^2 [5].

Fetal cells grow three to four times more rapidly than older skin cells cultured in the same manner, and these banked fetal cells are very resistant against physical and oxidative stress when compared to adult skin cells under the same culture conditions. Early-gestation fetal wounds heal without scar formation. Understanding the mechanism of this scarless healing may lead to new therapeutic strategies for improving adult wound healing. The fast reepithelialization and prompt presence of many fibroblasts in the fetal model suggest that rapid healing might play a role in scarless healing [6]. In China to explore the effects of bone marrow-derived mesenchymal stem cells (BMSCs) transfected with adenoviral vector carrying hepatocyte growth factor (HGF, Ad-HGF) was found to be effective in burn wound healing. A group of investigators who used transplantation of BMSCs modified with Ad-HGF concluded that it has positive effects on the healing of burn wounds probably through differentiation and release of relevant cytokines and this was found to be effective in the rodent model [7].

Conclusion

Adult (postnatal) skin wound healing is a complex and well-orchestrated process spurred by attendant inflammation that leads to wound closure with scar formation. In contrast, fetal wound repair occurs with minimal inflammation and faster reepithelialization and without the accumulation of scar [8]. But there are more problems if the patient reports late, thereby making it a chronic nonhealing burn ulcer where pediatric skin application may not make a complete scarless healing.

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28 Concern for Pharmacogenomics and Autologous Cell Therapy: Can This Be a Direction Toward Medicine for the Future?

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Personalized medicine is a rapidly expanding field offering patient-specific therapies to treat disease. Occasionally, the emergence of new technology or knowledge propels medicine across a threshold that is so monumental that it mandates changes in the structure of health-care delivery. Today, medical science is at another such threshold with the advent of individualized medicine. The Mayo Clinic Center for Individualized Medicine (CIM), for instance, is designed to discover and integrate the latest in genomic, molecular, and clinical science into personalized care for patients across a multiple-site academic medical center [1]. Major investments in basic science have created an opportunity for significant progress in clinical medicine. Individualized medicine has advanced because researchers have discovered hundreds of genes that harbor variations contributing to human illness and identified genetic variability in patients' responses to dozens of treatments and have begun to target the molecular causes of some diseases. In addition, scientists are developing and using diagnostic tests based on genetics or other molecular mechanisms to better predict patients' responses to targeted therapy [2]. Genome-wide association studies involve hundreds of thousands of single-nucleotide polymorphisms (SNPs) which are tested for association with a disease in hundreds or thousands of persons. The genome-wide association study has therefore revolutionized the search for genetic influences on complex traits [3, 4]. It has been discovered that many diseases are caused by many genetic and environmental factors working together, each having a relatively small effect [5].

Human genetic data are accumulating at an ever-increasing pace, and whole-genome sequences of individuals from multiple populations are now publicly available. The growing inventory of human genetic variation is facilitating the understanding of why susceptibility to common diseases varies among individuals and populations. In addition, insights are being gained that may improve the efficacy and safety of therapeutic drugs. Such knowledge is relevant to fundamental questions about our origins, differences, and similarities [6, 7]. It is a matter of great scientific interest that humans have about half the amount of genetic variation that is observed in Central African chimpanzees and gorillas [8] and about one tenth the amount of variation seen in the fruit fly *Drosophila pseudoobscura* [9]. The fact that we have less variation than many gorilla and chimpanzee populations, despite our much larger current population size, reflects prehistoric bottlenecks in human population size [10]. Some mammals, such as cheetahs, have also undergone population bottlenecks and have even less genetic diversity than humans [11].

Most common SNPs (i.e., those for which the prevalence of the less common allele is greater than 5 %) are shared among populations from different continents [12]. This commonality reflects continued migration and gene flow among human populations throughout history, in addition to our recent common origin. Disease prevalence, severity, and resistance vary considerably among ethnic groups as a consequence of inherited factors and noninherited causes, such as poverty, unequal access to care, lifestyle, and health-related cultural practices [13–15]. Well-known examples include the elevated prevalence of

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Tay–Sachs disease among persons of Ashkenazi Jewish ancestry [15] and sickle cell disease, thalassemia, and glucose-6-phosphate dehydrogenase deficiency in some populations of African descent [16].

Clinically relevant pharmacogenetic examples, mainly involving drug metabolism, have been known for decades, but recently, the field of pharmacogenetics has evolved into "pharmacogenomics," involving a shift from a focus on individual candidate genes to genome-wide association studies. Such studies are based on a rapid scan of markers across the genome of persons affected by a particular disorder or drug–response phenotype and persons who are not affected, with tests for association that compare genetic variation in a case–control setting [17]. There is great variation in drug–response phenotypes, and a "one size fits all" paradigm for drug delivery is considered flawed in current medical practice. Only 7 years ago, the approval of the breast cancer drug Herceptin created a stir both in the medical community and the popular press. It helped only about one in five women who took it. But the women who benefited from the drug turned out to have a mutation in their tumor cells that clearly differentiated them from nonresponders.

When California-based biotech giant Genentech began selling the drug along with a diagnostic test that could determine which patients would benefit from it, Herceptin became more than just another cancer drug. The scientific community became conscious that treatment could be tailored to an individual patient's genetic profile. The history of medicine is full of medications with unintended consequences; the ability to understand some of the underlying causes has been a recent development. To cite another example, in the 1950s, succinylcholine was used by anesthesiologists as a muscle relaxant during operations. However, about 1 in 2,500 individuals experienced a horrific reaction – respiratory arrest. Later research revealed that those individuals who suffered respiratory arrest had defects in both copies of cholinesterase, the enzyme required to metabolize succinylcholine into an inactive form. There are similar adverse effects with the antiangina drug perhexiline, which caused neural and liver toxicity in a subset of patients. Scientists later found that this toxicity occurred in individuals with a rare polymorphism of CYP2D6, an enzyme involved in the drug's metabolism. Similar questions may be raised whether a 75-mg dose of clopidogrel used for a 70-kg adult would be appropriate for a 20-kg child. Similarly, even with identical body weight, two adults may differ in their response to a fixed dosage of a drug. Interestingly, for an increasing number of drugs, this appears to be the case. For instance, two patients with similar clinical presentations given the same dose of the antiplatelet drug clopidogrel may respond differently – while one could be adequately protected against cardiovascular events, the other could experience a myocardial infarction due to inadequate therapeutic protection. What accounts for this difference? Genetics? The patient with inadequate therapeutic protection most likely has a polymorphism of CYP2C19 with decreased activity, so that this key enzyme cannot efficiently metabolize clopidogrel into its active metabolite [18]. Let us consider some additional and essential examples which the FDA has approved along with its companion diagnostic procedure to be employed in clinical practice. For example, a very common anticancer drug, mentioned earlier, which appears to be effective in remission of the clinical course of breast cancer is Herceptin (trastuzumab). This targets HER2 (human epidermal growth factor receptor type 2) to treat metastatic breast cancer. Its supportive diagnostic system is the HER2 immunohistochemistry test which is actually a HER2 gene-amplification test.

Another example is Erbitux (cetuximab) which targets EGFR (epidermal growth factor receptor) to treat metastatic colorectal cancer; here, EGFR immunohistochemistry test helps therapeutic support. The third example is Gleevec (imatinib) which targets the cell surface tyrosine kinase receptor [19].

Why do gene diagnostic tests help in the selection of specific drugs for specific diseases in specific individuals? As mentioned earlier, the emerging concept of pharmacogenomics deals with the technology that analyzes how genetic makeup affects an individual's response to drugs. It deals with the influence of genetic variation on drug response in patients by correlating gene expression or single-nucleotide polymorphisms with a drug's efficacy or toxicity. By doing so, pharmacogenomics aims to develop rational means to optimize drug therapy, with respect to the patients' genotype, to ensure maximum efficacy with minimal adverse effects. Such approaches promise the advent of "personalized medicine," in which drugs and drug combinations are optimized for each individual's unique genetic makeup [20–24], with the patient population showing large interindividual variability in drug response and toxicity.

All major classes of cardiovascular drugs have proven efficacy in the treatment and prevention of cardiovascular disease. Not uncommonly, these drugs have narrow therapeutic indexes that are influenced by genetic variation – a hallmark of drugs for which pharmacogenomic approaches are likely to provide substantial clinical benefit (ACE inhibitors, β-adrenoreceptor antagonists, statins, and β-agonists). Similar effects are noticed in antidepressant drug categories. Selective serotonin reuptake inhibitors and tricyclic antidepressants, given at standard doses, do not have the same effect on all patients; a substantial proportion of patients do not respond, respond only partially, or experience adverse drug reactions (ADRs). Other examples are the anticoagulant agents warfarin and clopidogrel which are two of the widely prescribed cardiovascular drugs with narrow therapeutic indexes.

It is known widely in pharmacological circles that drug concentrations in plasma can vary more than 600-fold between two individuals of the same weight on the same drug dosage. This variation can be of genetic, physiological, pathophysiological, or environmental origin, but a drug's absorption, distribution and metabolism, and interactions with its target can be determined by genetic differences. In general, genetic factors are estimated to account for 15–30 % of interindividual differences in drug metabolism and response.

There are several known genes which are largely responsible for variances in drug metabolism and response. The most common are the cytochrome P450 (CYP) genes, which encode enzymes that influence the metabolism of more than 80 % of current prescription drugs. Codeine, clopidogrel, tamoxifen, and warfarin are examples of medications that follow this metabolic pathway [25–27]. Patient genotypes are usually categorized into predicted phenotypes. For example, if a person receives one allele each from the mother and the father in the coding for the CYP2D6 gene, then that person is considered to have an extensive metabolizer (EM) phenotype. An extensive metabolizer is considered normal. Other CYP metabolism phenotypes include: intermediate, ultrarapid, and poor. In theory, each phenotype is based upon the allelic variation within the individual genotype. However, several genetic events can influence a same phenotypic trait, and establishing genotype-to-phenotype relationships can thus be far from consensual with many enzymatic patterns. For instance, the influence of the CYP2D61/4 allelic variant on the clinical outcome in patients treated with tamoxifen remains debated today. In oncology, genes coding for DPD, UGT1A1, TPMT, and CDA involved in the pharmacokinetics of 5-FU/capecitabine, irinotecan, 6-mercaptopurine, and gemcitabine/cytarabine, respectively, have all been described as being highly polymorphic. A strong body of evidence suggests that patients affected by these genetic polymorphisms will experience severe/lethal toxicities upon drug intake and that pre-therapeutic screening helps to reduce the risk of treatment-related toxicities through adaptive dosing strategies [28].

As mentioned earlier, and as is clear from the discussion so far, pharmacogenomics has applications in illnesses like cancer, cardiovascular disorders, depression, bipolar disorder, attention deficit disorders, HIV, tuberculosis, asthma, and diabetes as well as addiction to nicotine. However, there were no clear guidelines till very recently when the FDA produced a long-awaited guidance document clearly specifying what kinds of genomic data it will require for drug approvals and encouraging companies to submit preliminary data voluntarily in order to help build a scientific basis for interpreting pharmacogenomic experiments. The agency is now working with companies to develop another set of guidelines to define the process for validating biomarkers [28].

In cancer treatment, pharmacogenomic tests are used to identify which patients are most likely to respond to certain cancer drugs. In behavioral health, pharmacogenomic tests provide tools for physicians and caregivers to better manage medication selection and side effect amelioration. Pharmacogenomics is also known as companion diagnostics, meaning tests being bundled with drugs. Examples include KRAS test (GTPase KRAS) with cetuximab and EGFR test with gefitinib. Besides efficacy, germ line pharmacogenetics can help to identify patients likely to undergo severe toxicities when given cytotoxics showing impaired detoxification in relation with genetic polymorphism, such as canonical 5-FU [28]. In cardiovascular disorders, the main concern is response to drugs including warfarin, clopidogrel, beta-blockers, and statins. Many people take medications called SSRIs, or selective serotonin reuptake inhibitors, for different psychiatric disorders. Many of the medications are metabolized by CYP450 enzymes, including fluoxetine, paroxetine, and citalopram.

However, a difficult situation arises in case of autoimmune diseases which are mostly multifactorial in nature as in rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, type 1 diabetes mellitus, psoriasis, and inflammatory bowel disease; here, the epidemiologic, clinical, and therapeutic features cannot be explained with genetic data only. In each of these diseases, chronic and often intermittent inflammation contributes over time to the destruction of target organs that house inciting antigens or are the sites of immune-complex deposition. Although the adaptive immune system has long been a focus of attention, innate immune mechanisms are now viewed as being central to the pathogenesis of these disorders. In addition, the concept of quantitative thresholds for immune cell signaling has emerged in the past decade as a potential way of understanding how multiple genetic factors of relatively small effect may combine to create a state of susceptibility to autoimmune activation. New genetic findings have also indicated that the identification of the environmental components that interact with host genetic factors is critical in developing a deeper understanding of autoimmunity, as well as new approaches to prevention and treatment [29]. A special mention may be made of obesity and diabetes. Here, an improved understanding of pathophysiology achieved through genetic discovery provides new opportunities for diagnosis, treatment, and monitoring. Studies of risk variants for type 2 diabetes in healthy populations have shown that most variants act through perturbation of insulin secretion rather than insulin action, establishing inherited abnormalities of beta-cell function or mass (or both) as critical components of the progression to type 2 diabetes [30]. The time required to achieve clinical translation is often underestimated [31], and most of the discoveries in multifactorial disease have simply been too recent for their full translational potential to be realized.

That potential lies in three main areas: the characterization of disease mechanisms that provide new targets for treatment and prevention, improved risk prediction and differential diagnosis, and personalized treatment and prevention. In cases of monogenic disease, genetic information can provide powerful diagnostic and predictive value for selected patients. Since subtypes of monogenic diabetes and obesity vary in their prognostic implications and therapeutic recommendations, a definitive molecular diagnosis is an important component of clinical management [32, 33]. However, even next-generation sequencing technologies are likely to be transformative in the medium term, though distinguishing pathogenic mutation from incidental variation will remain a challenge.

The individual's genes are imprinted in his/her cells. One problem though, if a person has diabetes, will his own cells (presumably diabetic) help therapy? How do autologous cells help in the aetiopathogenesis of any of these systemic diseases, for example, SLE?

Apart from progressively increasing concern for pharmacogenomics, the most recent pillar in pharmacological science or health care is live cell-based therapy, popularly known as cell therapy; the other pillars are pharmaceuticals, biologics, and devices [34]. The current three main therapeutic pillars of health-care therapy are each built upon different platform technologies with unique core competencies: small molecules, biological molecules, and devices. Their different underpinning sciences – chemistry (pharma), molecular biology (biotech), and physics and engineering (medical devices) – define their resultant products and services. Cellular (cell) therapy can be defined as the use of cells to treat disease. Its origins can be traced to the early 1800s, when Dr. Charles-Edward Brown-Séquard (1817–1894) injected animal testicle extracts to stop the effects of aging. His therapy was very popular in the last years of the nineteenth century. He even had followers in the following century, among whom is Serge Voronoff (1866–1951), who grafted monkey testicles in replacement of human ones [35].

Modern cellular therapy obtained its scientific legitimacy mainly from the field of bone marrow transplantation. The spectrum of cell therapies is currently highly diverse but broadly has two major categories: permanent cell therapies such as the replacement of limbal cells for damaged corneas [36] and transient cell therapies such as the immunomodulation provided by adult stem cells for the treatment of graft-versus-host disease. In January 2009, Osiris Therapeutics, a leading stem cell therapeutics company, announced that the FDA had given clearance to broaden its expanded programme for Prochymal, making the stem cell product available to adults with Graft vs Host disease (GVHD) [37, 38]. The number of different types of cells used for therapies are expected to rapidly expand in the future with the potential for bespoke designer cells, synthetically engineered cells, cell fragments, cell hybrids, substitute tissues, and the gradual emergence of enhancement therapies rather than mere health restoration.

It may, however, be pointed out here that the type of stem cells used for therapy is important. Embryonic stem cells carry an increased risk of tumor formation, a characteristic not exhibited in autologous adult stem cells. Increased safety and decreased ethical controversy make autologous stem cells an appealing therapeutic option for neurological disease. The debate currently surrounding not only embryonic stem cell retrieval but also stem cell usage in general has sparked issues with its usage. Obtaining cells from a patient's own body would circumvent the ethical controversy as it is not harvesting viable cells from one individual donor to another individual recipient, but it is entirely for the same patient. Additionally, these cells would not be taken in ways possibly deemed as "therapeutic cell cloning," which is part of the current debate surrounding embryonic and fetal stem cells.

Autologous cell transplantation, therefore, may constitute a form of personalized medicine in the future that could afford many benefits in the clinical setting, such as the elimination of graft-versus-host disease. Transplantation of autologous stem cells may circumvent the need for immunosuppressants, which can cause many deleterious side effects to the patient. Increased availability is another advantage of autologous stem cell transplantation. Ethical controversy surrounding the collection of a patient's own stem cells is greatly reduced, thus increasing the accessibility of these cells. Unlike embryonic and fetal stem cells, harvesting autologous stem cells from the patient does not harm another organism.

Autologous cell therapy has gradually established itself as an important field of research with considerable progress in the last years. Several disorders, including those of inflammatory, traumatic, degenerative, and autoimmune nature, are listed as potential targets for stem cell application. While the bone marrow leads the investigations, other sources of stem cells have been explored, searching for cells with higher plasticity and tissues with facilitated harvesting [39]. Disposable tissues, such as the amniotic fluid, placenta, and, more recently, menstrual blood, are being investigated as potential sources of stem cells for therapy [40–42].

The immature phenotype, high proliferative potential, and immunomodulatory effects of these cells suggest that these are powerful tools for repair. Even when you have a genetic disease?

To cite an example, neurovascular diseases are the third leading cause of death in the United States and the first cause of chronic disability [43, 44]. Aging of the population and changes in lifestyle, especially in developed countries, con-

tribute to the progressive increase in the incidence of these disorders, more specifically stroke [45]. On the other hand, treatment is limited, and the only approved therapeutic agent for ischemic stroke is tissue plasminogen activator (tPA). More limiting, however, is the time window for tPA application, restricted to up to 3 h after symptom onset [46]. As a result, a report from 2008 estimated that only 1.8–2.1 % of all stroke patients had been treated with tPA in the United States [47]. It is clear, therefore, that therapeutic alternatives are warranted for the remaining stroke-affected patients which, excluded from tPA benefits, are exposed to the chronic consequences of the disease.

Menstrual blood cell injections are proposed as a restorative therapy after stroke, aiming to provide functional improvement and, therefore, decrease disability of the affected patients. Migration to the site of injury, immunomodulation, and secretion of neurotrophic factors are their main footholds as therapeutic agents. When compared to bone marrow-derived cells, menstrual blood cells present more immature phenotype and behavior, albeit maintaining the characteristic adult stem cell safety [48, 49]. Experimental studies have demonstrated benefits of menstrual blood cell administration, with tissue repair and functional improvement not only in the central nervous system, but also in the heart and ischemic limbs [50, 51]. Cell differentiation, although demonstrable in vitro, is still heterogeneously reported in the literature, and its relevance to the final outcome is not yet established. Of more importance may be the endogenous pathways of repair, which are also stimulated by the administrated cells.

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Introduction

Close to five million people in the USA are affected each year by chronic wounds and billions of dollars are spent annually for their treatment. Despite advances in chronic wound management over the past decades, many patients afflicted with chronic wounds fail to heal or their ulcers recur. Why is this so?

A chronic wound is a wound that does not heal in an orderly set of stages and in a predictable amount of time the way most wounds do; wounds that do not heal within 3 months are often considered chronic. Chronic wounds often remain in the inflammatory stage for too long. Due to loss of critical balance between production and degradation of molecules such as collagen and essentially the phase of degradation plays too large a role.

Acute and chronic wounds are at opposite ends of a spectrum of wound healing types. Chronic wound patients often report with ulceration, pain, poor circulation, neuropathy, repeated trauma, vasculitis (an inflammation of blood vessels), immunosuppression, pyoderma gangrenosum, and background diseases that can cause ischemia. Immunosuppression by drugs, for example, steroids, may play an important role apart from emotional stress, which can also negatively affect the healing of a wound, possibly by raising blood pressure and levels of cortisol, which lowers immunity.

Stem cells are unique primitive undifferentiated cells with transdifferentiation potential depending on the niche of the cell where it is placed or migrated due to injury. Mesenchymal cells have very little immunogenicity, thus making it the ideal candidate for cell therapy. Apart from bone marrow and adipose tissue, many new mesenchymal stem cell sources are now being identified. Recently, neonatal thymus-derived mesenchymal stromal cells (nTMSC) have been evaluated for the differentiation and immunomodulatory properties thereof and found to possess all the mesenchymal cell properties [1]. Menstrual blood stromal stem cells (MBSCs) have been demonstrated to exhibit stem cell properties such as the capability for self-renewal and multipotency, allowing for multilineage differentiation. In addition, this cell type has various immunomodulatory effects [2]. Similarly, adipose tissue belongs to another group which contains several types of stem and progenitor cells, including adipose tissue-derived stromal cells (ADSCs), endothelial progenitor cells, and hematopoietic and immune system cells. ADSCs share most of the phenotypical and functional characteristics of mesenchymal stromal cells (MSCs): bone marrow-derived mesenchymal stromal cells (BM-MSCs) or MSCs present in cord blood, placenta, and umbilical cord. The basic function of ADSCs is the preservation of the adipose tissue integrity by the production of adipocytes in the intensity proportional to their degradation. Recently, it has been proven that adipose tissue may contain more MSC-like cells than bone marrow (which serve as the gold standard of cells available for autologous cellular therapies).

Mesenchymal stromal cells (MSC) are promising candidates for cell therapy and tissue engineering and may be used to treat acute graft-versus-host disease (GvHD). However, major obstacles for their clinical use are the required cell dose and the biosafety and potential immunogenicity of fetal bovine serum (FBS), which is a crucial supplement of all media currently used for the culture of MSC [3]. Mesenchymal stromal cells are promising candidates for novel cell therapeutic applications. For clinical-scale manufacturing, human factors from serum or platelets have been suggested as alternatives to fetal bovine serum. It was previously shown that pooled human serum (HS) and thrombin-activated platelet releasate in plasma (tPRP) support the expansion of adipose tissue-derived MSCs [4].

Bone marrow-derived mesenchymal stem cells (BM-MSCs) can contribute to wound healing after skin injury. However, the role of BM-MSCs on repairing skin

appendages in renewal tissues is incompletely explored. There is emerging evidence that the use of bone marrow-derived mesenchymal stem cells can offset this situation of impaired healing [5]. However, most preclinical studies suggest that the therapeutic effects afforded by BM-MSC transplantation are short-lived and relatively unstable. Recent study suggested that the BM-MSCs may deliver epidermal growth factor (EGF) if placed inside a microsphere-based engineered skin model. Hence, it may be a promising strategy to repair sweat glands and improve cutaneous wound healing after injury and success in this study might provide a potential benefit for BM-MSCs administration clinically [6]. We all know that chronic wounds are common. In case of treatment for disabling chronic wound conditions, the benefit of bone marrow-derived mesenchymal stem cells in wound healing appears to be rewarding. Using an excisional wound splinting model, it was shown that injection around the wound and application to the wound bed of green fluorescence protein (GFP)(+) allogeneic BM-MSCs significantly enhanced wound healing in normal and diabetic mice compared with that of allogeneic neonatal dermal fibroblasts or vehicle control medium. Fluorescence-activated cell sorting analysis of cells derived from the wound for GFP-expressing BM-MSCs indicated engraftments of 27 % at 7 days, 7.6 % at 14 days, and 2.5 % at 28 days of total BM-MSCs administered. BM-MSC-treated wounds exhibited significantly accelerated wound closure, with increased reepithelialization, cellularity, and angiogenesis [7]. Chronic wounds of the skin may affect only the epidermis and dermis, or they may affect tissues all the way to the fascia. Ischemia is an important factor in the formation and persistence of wounds, especially when it occurs repetitively (as it usually does) or when combined with a patient's old age [8]. Ischemia causes tissue to become inflamed and cells to release factors that attract neutrophils such as interleukins, chemokines, leukotrienes, and complement factors. Next important is the bacterial colonization. Since more oxygen in the wound environment allows white blood cells to produce ROS to kill bacteria, patients with inadequate tissue oxygenation, for example, those who suffered hypothermia during surgery, are at higher risk for infection. The host's immune response to the presence of bacteria prolongs inflammation, delays healing, and damages tissue. Infection can lead not only to chronic wounds but also to gangrene, loss of the infected limb, and death of the patient. Another important factor is the viability of growth factor and the proteolytic enzyme at the site of the wound.

Chronic wounds also differ in makeup from acute wounds in that their levels of proteolytic enzymes such as elastase [9] and matrix metalloproteinases (MMPs) are higher, while their concentrations of growth factors such as platelet-derived growth factor and keratinocyte growth factor are lower. Since growth factors (GFs) are imperative in timely wound healing, inadequate GF levels may be an important factor in chronic wound formation. It may be noted that the art of healing is very complex. Scientists are increasingly curious to see whether the dynamic mesenchymal cell has a positive impact on the healing of a cutaneous wound which requires a well-orchestrated integration of the complex biological and molecular events of cell migration and proliferation and extracellular matrix (ECM) deposition, angiogenesis, and remodeling [10]. Diabetes constitutes an important segment of nonhealing wound. Diabetic foot ulcers are more likely to be of neuropathic origin and therefore eminently preventable; however, if not adequately taken care of, they become nonhealing chronic wounds [11].

One type of stem cell which has been isolated from a number of tissues and organs is the stromal stem cell which is similar in morphology and phenotype to the bone marrow-derived stromal cell termed mesenchymal stem cell. Human mesenchymal stem cells have effective multilineage potential [12] given the niche it could be differentiated into human hepatocytes without fusion [13]. If placed in the heart, it would differentiate into a cardiomyocyte phenotype in the adult murine heart [14, 15]. Similarly, if placed in the neuronal environment of the brain, these marrow stromal cells migrate throughout the forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains [16, 17]. However, MSC populations from different origins display some differences in terms of their patterns of gene expression and their differentiation capacity. There is an interesting article in this book by Prof. Parolini. She emphasized that such differences might be the consequence of at least two factors. The first of these may be considered "operational," given that most of the information available on the phenotype and functional properties of MSCs is derived from studies performed on cells cultured in vitro; however, the culture conditions themselves may give rise to the selection of different cell populations and may also induce heritable and epigenetic cellular preconditioning, thereby altering the original cellular phenotype [18]. The initial applications for which MSCs have been used in therapy are based on their absent or low immunogenicity and their immunoregulatory functions, as well as their multilineage differentiation capacity. Indeed, on one hand, a major advantage of using human MSCs for in vivo therapies is the fact that these cells are considered to be "immunoprivileged," due to their low expression levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I and their negative expression of major MHC II and co-stimulatory molecules such as CD40, CD80, and CD86. Therefore, allogeneic transplantation of MSCs should not require immunosuppression of the host. In addition, several evidences also show that MSCs may play specific roles in immunomodulation, interacting with cellular components of the immune system and inducing a shift from the production of pro- to anti-inflammatory cytokines [19].

Amniotic Fluid and Amniotic Membrane: A New Mesenchymal Source of Cell Therapy

Cell therapy describes the process of introducing new cells into a tissue in order to treat a disease. For conventional use, bone marrow autologous cells and adipose tissue autologous cells are conventionally used for cell therapy purposes. In the present study, the material used for cell therapy is freshly collected amniotic fluid and the amniotic membrane from women admitted by the family planning department in a government hospital for hysterotomy and ligation. Under normal circumstances, the amniotic membrane and the amniotic fluid are immediately disposed off and eventually incinerated. These materials are discarded everywhere, although they appear to have major therapeutic potentials for the successful treatment of burn wounds.

To recapitulate, amniotic fluid is to be found in the amniotic cavity that protects the fetus as a buffer and also helps growth and movement and prevents adherence to the placenta or the surrounding structures. This clear watery fluid is contributed principally from the maternal blood via the amniotic fluid epithelium but freely intermixes with secretions from the fetal lung, kidney, gastrointestinal tract, and skin; hence, the properties of this specialized fluid compartment are quite complex because of the contributions of both the maternal and the fetal components. In this connection, it may be mentioned that the amniotic cavity is delimited by the amniotic epithelium, the chorion laeve, and the decidua capsularis. The main constituents are water and electrolytes (99 %) together with glucose, lipids from the fetal lungs, proteins with bactericide properties, and fetal epithelium cells. As mentioned earlier, pluripotent progenitor cells isolated from the amniotic fluid and the placenta possibly present an exciting contribution to the field of stem cell biology and regenerative medicine. Compared with embryonic stem cells, progenitor cells isolated from the amniotic fluid have many similarities: they can differentiate into all three germ layers, they express common markers, and they preserve their telomere length. However, progenitor cells isolated from the amniotic fluid and placenta have considerable advantages.

They easily differentiate into specific cell lineages, and further, they avoid the current controversies associated with the use of human embryonic stem cells.

Pregnancy results in the acquisition of specialized and unique cells that may have clinical applications and therapeutic potential. Whether the pregnancy-associated progenitor cells (PAPCs) are hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), or a new population of stem cells is an unresolved issue. It is also unknown whether PAPCs respond to all types of maternal injury or only those injuries that recruit stem cells. It is possible that these cells, since they are fetal in origin, have a higher proliferative capacity or more plasticity than their equivalent adult (maternal) cells. In the current debate over the use of embryonic stem cells for the treatment of disease, the discovery of a population of fetal stem cells that apparently differentiate from the ones in adult women, and can be acquired without harming the fetus, may be significant [20, 21]. The growing fetus in the womb is a good source of stem cells. The initial interest in the field started with the use of placental blood-derived hematopoietic stem cells in Fanconi anemia in 1988 by the legendary Prof. Elaine Gluckman. Meanwhile, scientists have been able to isolate and differentiate only 30 % of mesenchymal stem cells (MSCs) on average, extracted from a newborn's umbilical cord jellylike material, shortly after birth. The success rate for amniotic fluid-derived stem cells, on the other hand, is close to 100 %. Analysis of surface markers shows that progenitor cells from amniotic fluid express human embryonic stage-specific marker SSEA4 and the stem cell marker Oct4 and do not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, and TRA1-81.

Differentiation of Amniotic Fluid- and Placenta-Derived Progenitor Cells

The progenitor cells derived from amniotic fluid and the placenta are pluripotent and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, hepatic, and renal phenotypes in vitro. Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type of interest. In 2007, Perin et al. [22] showed that AFSC (amniotic fluid stem cells) could be induced to differentiate into renal cells when placed into an in vitro embryonic kidney environment. In this preliminary clinical study, freshly collected amniotic fluid has been utilized as a source of cell therapy with the hypothetical assumptions that the mesenchymal cells of the AF (amniotic fluid) will participate in the repair process, the viscosity of the amniotic fluid will assist lubrication, and the bactericidal property of the amniotic fluid will guard against inadvertent infection.

Result and Analysis

After admission, all the cases, as shown in attached photographs, were treated with (1) normal saline for initial removal of the dirt and debris followed by (2) sprinkling of copious amounts of freshly collected clear amniotic fluid at the site of the wound (after 5–10 min) and, lastly, the application of amniotic membrane (3) at the wound site (amniotic or the fetal side of the amniotic membrane in case of superficial or partial thickness skin wound for early epithelialization and maternal attachment site or the chorionic site in deep wound to improve circulation through angiogenesis-supporting cytokine content of the chorionic site of the membrane).

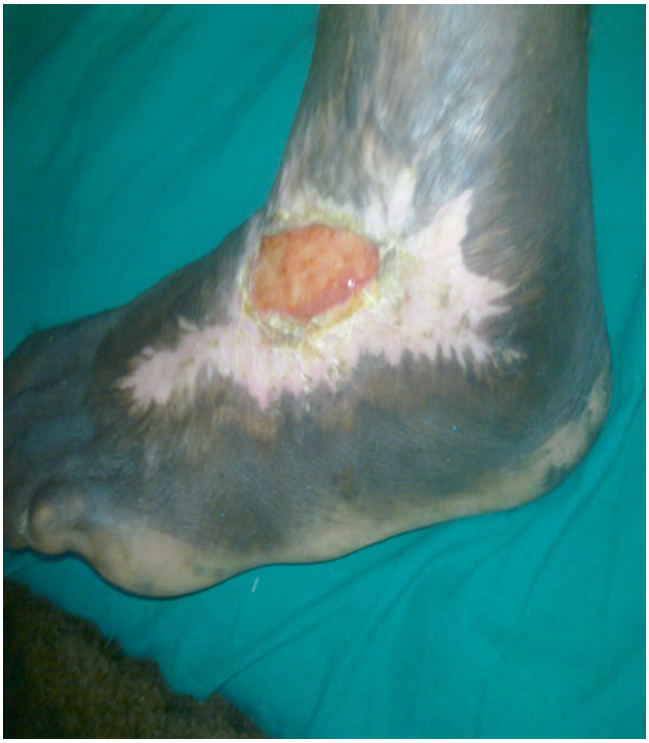

Fig 29.1 A chronic nonhealing burn ulcer treated with amniotic membrane

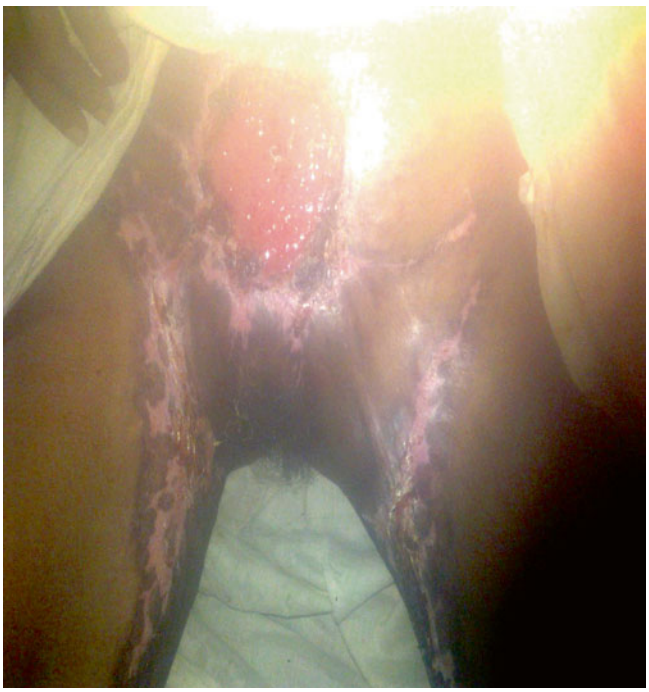

Fig 29.2 A chronic nonhealing burn ulcer treated with amniotic membrane

This amniotic membrane was kept in the amniotic fluid, which was freshly collected from consenting donor mothers who were VDRL-, hepatitis B and C-, and HIV (1 and2)-negative and had undergone cesarean section.

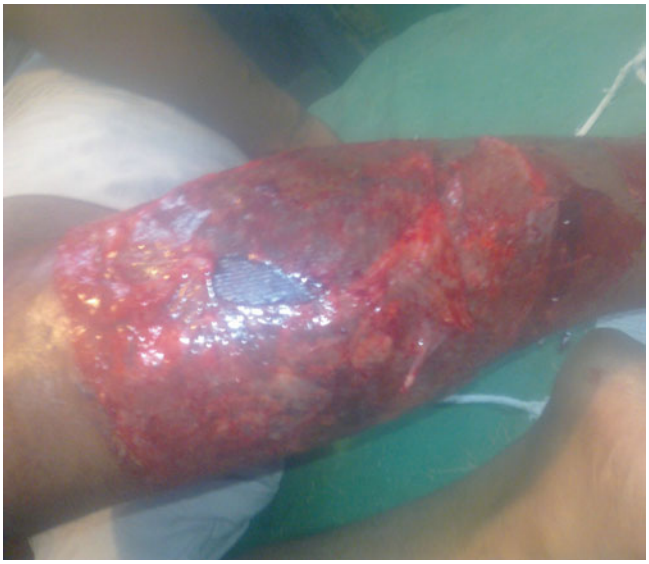

Fig 29.3 A chronic nonhealing burn ulcer treated with amniotic membrane

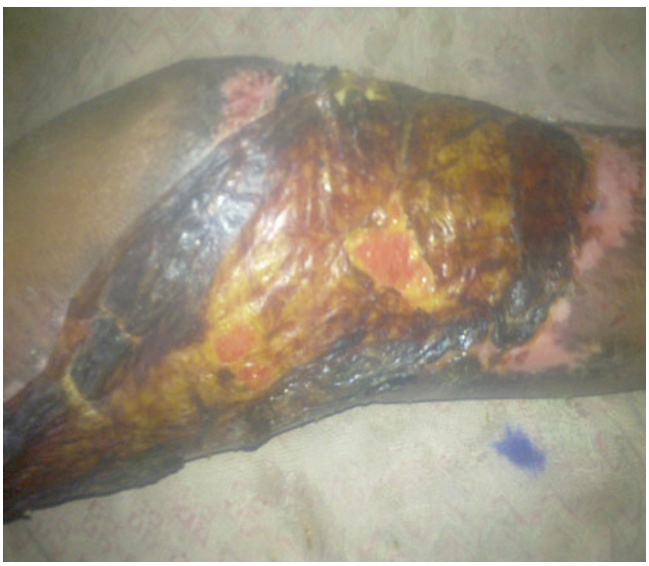

Fig 29.4 A chronic nonhealing burn ulcer treated with amniotic membrane

In this connection, photographs (Figs. 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, and 29.8) of patients with common postburn nonhealing ulcers persisting from 3 to 6 months with varying backgrounds who were admitted in our hospital have been shown; the burn wounds were treated regularly with freshly collected and screened amniotic membrane (for hepatitis B, C, HIV1 and 2, etc.), the goal being healing. The response in all cases was positive. This is a preliminary communication where we wanted to emphasize the utility of these pregnancy-specific biological substances like amniotic membrane and amniotic fluid dressing in augmenting the healing process of the wound.

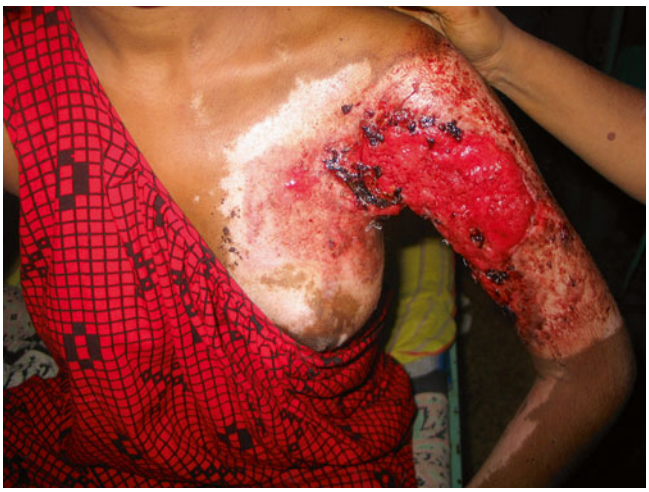

Fig 29.5 A chronic nonhealing burn ulcer treated with amniotic membrane

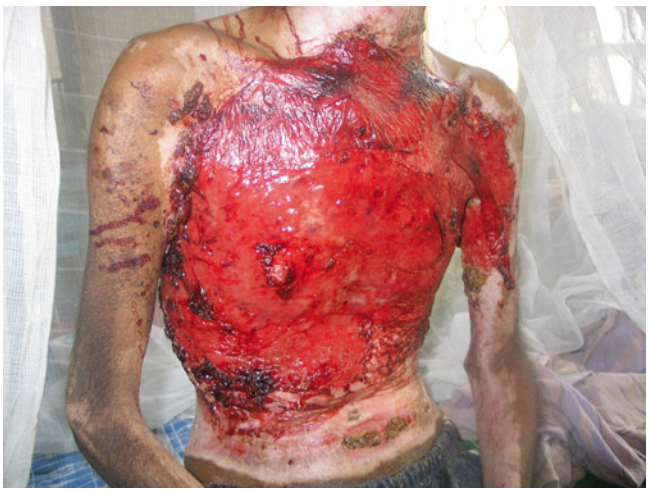

Fig 29.7 A chronic nonhealing burn ulcer treated with amniotic membrane

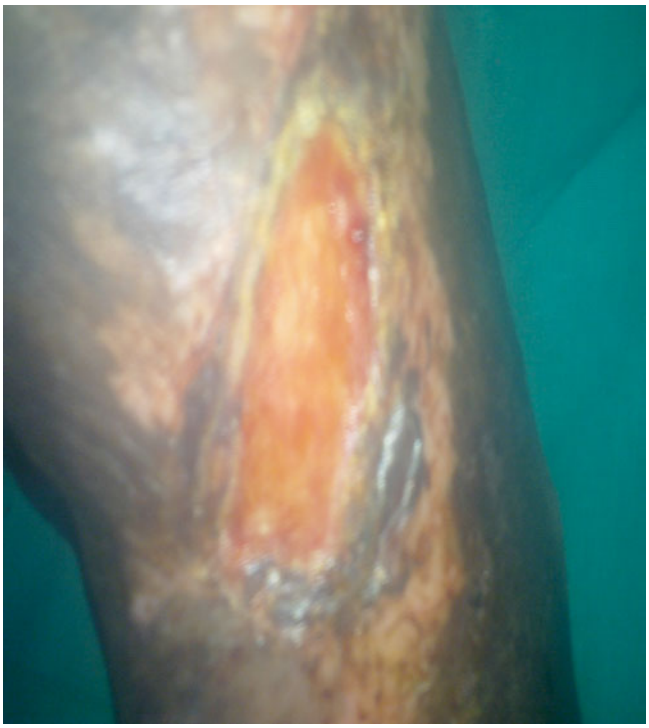

Fig 29.6 A chronic nonhealing burn ulcer treated with amniotic membrane

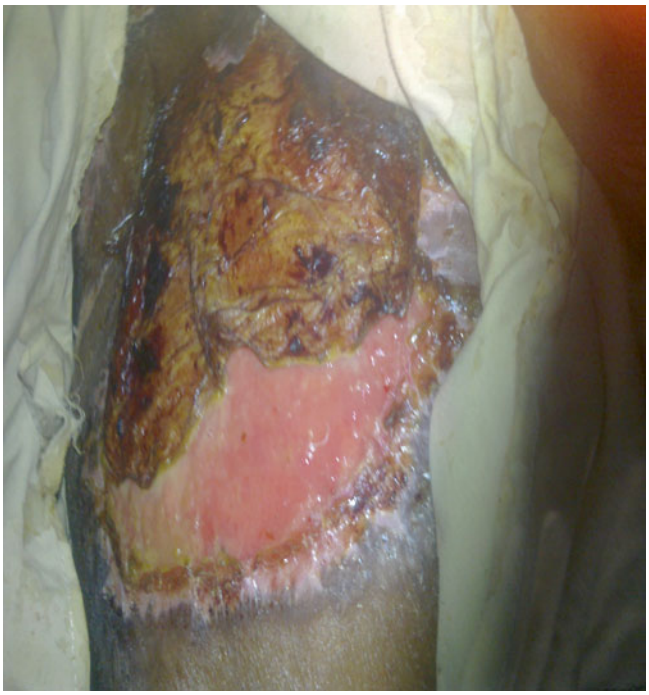

Fig 29.8 A chronic nonhealing burn ulcer treated with amniotic membrane

Discussion

Knowledge about the mechanisms of action of the cells on the injured site has changed over the years, contributing to advances in their application. Firstly, cell differentiation may not be necessary to promote repair. This observation implies that cells derived from adult tissues, which do not usually differentiate as easily as the pluripotent cells from more immature tissues, may be used. Second, regeneration may occur without cell migration to the site of injury. This finding implies the utility of intravenous applications of the cells, instead of the more invasive local injections. Today, it is established that stem cells may be derived from numerous tissues, with some variability in their therapeutic potentials.

There is an interesting animal study which demonstrates systemically the beneficial effect of BM-MSCs in cutaneous regeneration and wound healing in nondiabetic and diabetic mice through differentiation and paracrine effects. Administration of allogeneic BM-MSCs or BM-MSC-

derived molecules may represent novel therapeutic approaches in the treatment of chronic wounds and other conditions [23]. The regenerative mechanisms of mesenchymal stromal cell (MSC) grafts include direct commitment toward differentiating cells together with paracrine communication with resident connective cells and infiltration of inflammatory cells, antigen-presenting cells, or both [24]. Paracrine interactions require these cells to produce and respond to a variety of trophic factors that may stimulate the resident cells to differentiate and themselves renew the pathological tissue [25]. In addition, MSCs also exhibit immunomodulatory functions, making them a potential tool to combat an immuno-infectious disease such as periodontitis. The reduction of inflammation by MSCs may halt the development of injury and allow regenerative processes to take place [26]. Furthermore, MSCs may exert a neovascularization effect [27]. This is possibly the basis for the augmented strategy of healing with the application of pregnancy-specific biological substances as dressing material. The therapeutic effect of the stem cell seems consistent with both the paracrine function and the transdifferentiation. Systemic and micro-milieu factors appear to dictate the fate of implanted stem cells. Researchers must begin to focus upon a few basic critical issues: the modulation of the systemic and microenvironment for stem cells in order to augment stem cell survival and transdifferentiation, the underlying mechanisms of stem cell therapy and the fate of stem cells, and the differentiation into specific cell types as per local demand or other terminal cell populations with synchronizing and favorable paracrine functions. Earlier scientists used stem cells in different routes, namely, subcutaneous or intravenous route. In the present study, the transdermal route, which is seen as deficient, was used for fetal and neonatal stem cell-studded pregnancy-specific biological substances (PSBS) like the placenta, amniotic fluid, and the membrane (as burn dressing), with good effect in burn victims.

This is the clinical validation of the fetal and neonatal stem cell-studded pregnancy-specific biological substances, which are used through the deficient transdermal route in burn victims.

In the earlier paragraphs, we have discussed in brief the major advances in the field of PSBS and its potential regenerative impact through the progenitor or stem cells found in it. This property has made them a unique category on the tissue used for regenerative biology. The present article is a report on the simultaneous and judicious utilization of the chorionic (for angiogenesis) and amniotic membrane (for epithelialization) and is a clinical validation of molecular advances in the field of stem cell biology in the case of burn victims.

The utilization of bio-friendly amniotic fluid with intrinsic bactericidal property instead of traditional normal saline, not only reduces the unnecessary utilization of costly antibiotic cream in dressing, but also helps in the prevention of the emergence of bacterial resistance and mixed infection due to opportunistic bacteria, fungi etc.

Conclusion

Thus, one of the therapeutic functions of MSCs is the early induction of granulation tissue followed by the stabilization of the neovascular network [24, 28]. If we come back to our study, it is worth remembering that the skin offers a perfect model system for studying the wound healing process, which involves a finely tuned interplay between several cell types, pathways, and processes. The dysregulation of these factors may lead to wound healing disorders, resulting in chronic wounds, as well as abnormal scars such as hypertrophic and keloid scars. In the present work, the surmise is that mesenchymal and epithelial stem cells supplied by freshly collected and screened amniotic fluid and placental membrane, are possibly, in combination, acting as a cell therapy source to augment the process of healing.

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Ethics and Moral Principles in the Practice of Medicine

30

Sanjukta Bhattacharya, Phillip G. Stubblefield, Sushanta Kumar Banerjee, and Niranjan Bhattacharya

Medical ethics is a system of moral principles that applies morality and value judgments to the practice of medicine. Just like any other branch of ethics, there can be no universal code of value judgments because these depend on cultures and cultures differ in practice of daily life. What may be judged as common practice in one culture may be considered totally unethical in another. For instance, widow remarriage is part of the Christian and Islamic cultures from the very beginning but was forbidden and considered unethical as well as illegal leading to ostracization in the Hindu culture till the British brought their own (Christian) culture to prevail in India. However, certain tenets of ethics are applicable across the board even though there may be deviant groups who believe in just the opposite, and among them, one of the most important is the sanctity of life. Medical ethics encompasses the sanctity of life and all that goes with it. The dilemma that sometimes arises is the same as those which appear at the beginning of any new breakthrough in any branch of science: there are those who use religion and ethics to castigate the few immensely gifted people who think beyond the box and come up with new ideas that take us closer to universal truths. Take, for instance, the case of Galileo Galilei: his views on heliocentrism were not accepted during his lifetime, when most subscribed to geocentrism or the Tychonic system. The Roman Inquisition investigated the matter in 1615 and found heliocentrism to be false because it was contrary to the scriptures. He was also tried by the Holy Office and found "vehemently suspect of heresy," forced to recant, and spent the last years of his life under house arrest. Today, however, he is known as the Father of Modern Science. Even the Catholic Church, which had once hounded Galileo, 300 years down the line, recanted and endorsed his theories. In 1939, Pope Pius XII in his first speech to the Pontifical Academy of Sciences acknowledged Galileo as being among the "most audacious heroes of research …not afraid of the stumbling blocks and risks on the way, nor fearful of funereal monuments" [1]. Similarly, it is well known that those pioneers of medicine, who had wished to study the human body to get an understanding of its workings, had to dig up dead bodies from graves in the middle of the night since dissecting cadavers was considered unethical. Later, during the Renaissance, with more enlightenment, human bodies were dissected. Leonardo da Vinci, best known as an artist and perhaps the ultimate Renaissance man, was given permission to dissect human corpses at Florence, Milan, and Rome, and he wrote a book on theoretical anatomy, with near-perfect sketches of human body parts. But interestingly, this book was not published at the time – it was published 161 years later, and that too under the title *Treatise on Painting*. A more appropriate title would have been "A Treatise on Anatomy," but perhaps the exact representations of the human body were not palatable to some and hurt the moral principles of others. Actually, da Vinci's anatomical knowledge came from postmortem dissection work that the church forbade. Yet, the sketches were so good that in 2005, a UK heart surgeon, Francis Wells, from Papworth Hospital, Cambridge, pioneered repair to damaged hearts using Leonardo's depiction of the opening phase of the mitral valve to operate without changing its diameter allowing an individual to recover more quickly [2]. The point that is being made here is that while the sanctity of life is at the

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base of all ethics, we may still have been in the dark ages if some brave men had not dared to challenge existing knowledge (without violating the sanctity of life), despite ridicule, harassment, incarceration, and even execution, in their search for newer and newer truths. The tension between what is considered to be "moral principles" at any given time or place and the search for scientific truth that will help humanity to go forward is not new and will not end in the near future, because as something is seen as theoretical at a certain point of time (and therefore attracts value judgments) and is proved beyond doubt as empirical science at a later date, the boundaries of ethics get stretched as the unacceptable becomes fact.

An interesting point to note in Western medical ethics as well as in Islamic and Jewish traditions is the prevalence of religious scholars in the early years in the decision of what was moral and what was not in medical practice. Historically, Western medical ethics may be traced to guidelines on the duty of physicians in antiquity, such as the Hippocratic oath, and early Christian teachings. The first code of medical ethics, *Formula Comitis Archiatrorum*, was published in the fifth century, during the reign of the Ostrogoth king, Theodoric the Great. In the medieval and early modern period, the field is indebted to Islamic scholars such as Ishaq ibn Ali al-Ruhawi (who wrote the *Conduct of a Physician*, the first book dedicated to medical ethics) and Muhammad ibn Zakariya ar-Razi (known as Rhazes in the West), Jewish thinkers such as Maimonides, Roman Catholic scholastic thinkers such as Thomas Aquinas, and the case-oriented analysis (casuistry) of Catholic moral theology. These intellectual traditions continue in Catholic, Islamic, and Jewish medical ethics, and religious issues still appear to deeply influence value judgments regarding medical research in the West. However, medicine was itself a part of the Vedas in Indian medicine, and apart from the *Atharva Veda*, there are more direct treatises on medicine like the *Sushruta Samhita* and the *Charaka Samhita*. The *Sushruta Samhita* discusses "surgical techniques of making incisions, probing, extraction of foreign bodies, alkali and thermal cauterization, tooth extraction, excisions, and trocars for draining abscess draining hydrocele and ascitic fluid, the removal of the prostate gland, urethral stricture dilatation, vesiculolithotomy, hernia surgery, caesarian section, management of haemorrhoids, fistulae, laparotomy and management of intestinal obstruction, perforated intestines, and accidental perforation of the abdomen with protrusion of omentum and the principles of fracture management, viz., traction, manipulation, appositions and stabilization including some measures of rehabilitation and fitting of prosthetics." Both the Sushruta and the Charaka Samhita were translated into Arabic language during the eighth century. The translator of the Sushruta Samhita was one Ibn Abillsaibial. The work was known as *Kitab Shah Shun al-Hindi* in Arabic, or alternatively as *Kitab i-Susurud*. The ninth-century Persian physician Rhazes was familiar with the text. It is known that the Arabic translation entered Renaissance Italy and helped in the development of modern medicine. What is important here is that the Indian tradition was not a Hindu tradition and medicine was apparently not restricted by religious value judgments. The human anatomy was studied, and however meager may have been the knowledge, the idea was to bring about a cure through the then known sciences. It is because of this approach perhaps that the *Samhita* went on to explore the basics of plastic surgery, at a time when Western religious scholars may have thought such subjects to be a taboo, if they were thought of at all (the text of the *Samhita* goes back to the third/fourth centuries AD). The *Samhita* lays down the basic principles of plastic surgery by advocating a proper physiotherapy before the operation and describes various methods or different types of defects, viz., (1) release of the skin for covering small defects, (2) rotation of the flaps to make up for the partial loss, and (3) pedicle flaps for covering complete loss of skin from an area. The author has mentioned various methods including sliding graft, rotation graft, and pedicle graft. Reconstruction of a nose (rhinoplasty) which has been cut off using a flap of skin from the cheek has been described. Labioplasty too has received attention in the *Samhita* [3]. As mentioned earlier, dissection of human cadavers was largely forbidden in the Western world even as late as the sixteenth century as per religious ethics, while this did not seem to be a problem in India, something that can be deduced from the kind of surgical procedures described in the *Samhita*. Thus, ethics and moral principles vary over time and place depending on the culture of the time and place. The main concern of medical ethics should be to do good while doing no harm to the individual's and the community's health.

It was only in the eighteenth and nineteenth centuries in Western Europe that medical ethics emerged as a more self-conscious discourse. In England, Thomas Percival, a physician and author, crafted the first modern code of medical ethics. He drew up a pamphlet with the code in 1794 and wrote an expanded version in 1803, in which he coined the expressions "medical ethics" and "medical jurisprudence" [4]. In 1847, the American Medical Association adopted its first code of ethics, with this being based in large part upon Percival's work. In the twentieth century, there was some shift away from Catholic moral values to Protestant liberal values articulated in thinkers like Joseph Fletcher. In the 1960s and 1970s, using liberal theory and procedural justice as a base, the idea and discourses of medical ethics reconstituted itself into bioethics. This has also to be seen in the context of actual experimentation on humans that violated the sanctity of human life, not for the good of the individual or the community, but largely for gaining an insight on how to enhance power in a bipolar world. Among such experiments were human radiation experiments, radioactive

experiments, and plutonium injections, to mention a few [5]. While the Nazis have been demonized for their bizarre experiments in pursuit of producing Germanic "super race" clones, the Americans too should be vilified for experiments on African-Americans from the time of slavery to the Tuskegee syphilis study which was conducted between 1932 and 1972, in which Black Americans suffering from syphilis were prevented from receiving the required medicine so that the evolution of the disease could be observed [6]. Because of the public outcry following the grossly unethical objectives and uninformed nature of the experiments conducted, which sometimes involved mentally disabled children, uninformed African-Americans (which seemed to be part of the racism that prevailed in America till well into the 1960s), prison inmates and guards (the Stanford prison experiment; the BBC prison study), and others similarly placed like the poor, powerless, and sick, medical ethics began to be foregrounded. But the crass nature of the experimentations prior to better judgment kicking in must be remembered. In Nashville, pregnant women were given radioactive mixtures. In Cincinnati, some 200 patients were irradiated over a period of 15 years. In Chicago, 102 people received injections of strontium and cesium solutions. In Massachusetts, 57 developmentally disabled children were fed radioactive oatmeal in an experiment sponsored by MIT and the Quaker Oats Company. In none of these cases were the subjects informed about the nature of the procedures, and thus they could not have provided informed consent [7].

Given this background, medical ethics, as it began to emerge, largely followed the "four principles" approach as set forth by Tom Beauchamp and John Childress in their book, *Principles of Biomedical Ethics* [8]. These are (a) *Voluntas aegroti suprema lex* (the principle of autonomy or the right of patients to choose their own treatment), (b) *Salus aegroti suprema lex* (beneficence – the medical practitioner should act in the best interests of the patient), (c) *primum non nocere* (non-maleficence or to "do no harm"), and (d) fairness and equality (justice in the distribution of scarce health resources and regarding who gets what treatment). Autonomy and beneficence may come into conflict when a patient disagrees with health care providers regarding the patient's best interests. Different societies differ in the ways that the conflict is settled with some societies prioritizing beneficence over autonomy, and here surrogate decision makers can play a role. Informed consent and confidentiality are two other principles of medical ethics that are important in modern day medical practice.

Another area of medical ethics and moral principles that has to be mentioned is the human rights regime and the debate that inevitably followed the imposition of the human rights regime. Most codes of ethics require the protection of the human rights defined in the Universal Declaration of Human Rights (1948) of the patient. Following this, in 1997, the Council of Europe adopted the European Convention on Human Rights and Biomedicine to create a uniform code on bioethics for its 47 members. This treaty entered into force only on 1 December 2009. As of 2013, 29 members had acceded to the convention. This convention provides protection for those unable to consent including children; for instance, it notes that "No organ or tissue removal may be carried out on a person who does not have the capacity to consent under Article 5." It also has protocols on cloning, transplantation, biomedical research, and genetic testing for health purposes. Article 2 is important in all contexts: "The interests and welfare of the human being shall prevail over the sole interest of society or science" [9].

Regarding the additional protocol on biomedical research, Article II is pertinent to this book as well as to all research in biomedicine, so much so that it may be worth quoting the entire content:

Article 3 – Primacy of the human being

The interests and welfare of the human being participating in research shall prevail over the sole interest of society or science.

Article 4 – General rule

Research shall be carried out freely, subject to the provisions of this Protocol and the other legal provisions ensuring the protection of the human being.

Article 5 – Absence of alternatives

Research on human beings may only be undertaken if there is no alternative of comparable effectiveness.

Article 6 – Risks and benefits

1. Research shall not involve risks and burdens to the human being disproportionate to its potential benefits.
2. In addition, where the research does not have the potential to produce results of direct benefit to the health of the research participant, such research may only be undertaken if the research entails no more than acceptable risk and acceptable burden for the research participant. This shall be without prejudice to the provision contained in Article 15 paragraph 2, sub-paragraph ii for the protection of persons not able to consent to research.

Article 7 – Approval

Research may only be undertaken if the research project has been approved by the competent body after independent examination of its scientific merit, including assessment of the importance of the aim of research, and multidisciplinary review of its ethical acceptability.

Article 8 – Scientific quality

Any research must be scientifically justified, meet generally accepted criteria of scientific quality and be carried out in accordance with relevant professional obligations and standards under the supervision of an appropriately qualified researcher [10].

The UNESCO too has adopted a similar convention, the Universal Declaration on Human Rights and Biomedicine, which also emphasizes that "In applying and advancing scientific knowledge, medical practice and associated technologies, human vulnerability should be taken into account. Individuals and groups of special vulnerability should be protected and the personal integrity of such individuals respected" [11]. What is worth noting is that the European experience of subjecting vulnerable populations to medical experiments underlies many of these conventions and protocols. However, the universal truth that medical research should be based on beneficence and non-maleficence is overarching in the moral principles set forth in these conventions. In current practice, much of this is ensured with steps like hospital accreditation and ethical guidelines set by ethical committees at the national, state, and hospital/institutional levels. There are provisions for legal actions to be taken in case of noncompliance with ethical mores of the state. However, as noted in the beginning, these mores may not be universal and may differ from culture to culture, and religion still plays a role in value judgments regarding what is ethical in medical practice and what is not.

The present book focuses on regenerative medicine using non-fetal sources like stem cells derived from mother's milk, menstrual blood, abdominal fat, adipose tissue, dental tissue, etc. Here, ethical issues regarding the donor will involve confidentiality and informed consent. There is no question regarding any harm being done to the donor. However, when the term "regenerative medicine" is used, the words that immediately cause controversy are "stem cell." There appears to be two grave misconceptions in the general public regarding stem cells – that it can cause miracles and that use of stem cells is unethical because harvesting stem cells can destroy human embryos and thus kill life before birth. Both ideas are not fully correct: the first because only the potentials of stem cells are yet being explored and cooperation from government, following all reasonable ethical principles, will make them as miraculous as projected and the second because here, critics are only talking of one type of stem cells, embryonic stem cells – isolation of somatic stem cells and induced pluripotent stem cells, however, do not require the destruction of embryos. Stem cells are available in adult fat, menstrual blood, and breast milk, and these too have the capability to regenerate tissue, but do no harm to the donor, and so far, potentially and in actuality in some cases where such matter has been used, the recipient in certain diseases. Thus, the field of regenerative medicine encompasses more than the embryonic stem cell controversy, and in terms of medical ethics and moral principles, it is time that scientists moved beyond the controversy to explore how different types of stem cells can help to regenerate lost or diseased tissue and thus help humankind in the long run.

Regenerative medicine is the medicine of the future. If one were to think clearly, nature has actually provided solutions to problems within the system that it has created in all spheres. Why then should the human body be an exception? Solutions to diseases may be found within the human system and the human system would probably provide the best cure thinkable. One of the fears in stem cell research is that nature would not know when to stop regenerating and there could be cancerous growth. Here, an example may be cited from the salamander. No other animal with a backbone can regenerate a lost limb. Yet the salamander can regenerate a lost leg or tail in just weeks. Recently, researchers discovered that salamanders do not convert adult cells all the way back to stem cells as originally expected. Instead, the salamander only partially reprograms cells at the site of an injury. Such a finding makes sense. As mentioned, one of the major problems with stem cells is their tendency to form cancerous growths. Cells that turn back the developmental clock only partially, instead of fully resetting the clock to an embryonic state, are less likely to turn cancerous. Even though a human being does not regenerate a lost limb, regrowth of tissue regularly happens within the human body. Every 2 weeks you change your skin. Every 10 years you replace your bones. The human liver can regenerate as long as one quarter of the organ remains. Researchers in the field of regenerative medicine hope to expand the human body's ability to repair itself. In some cases, doctors might be able to use biologically active molecules to stimulate tissue regeneration. In other cases, doctors might take a small amount of a patient's own tissue and use it to grow a replacement organ in a laboratory. Researchers at Wake Forest University in North Carolina have already used this approach to grow new bladders for spina bifida patients. The ability to grow organs in the laboratory from a patient's cells solves two of the current problems with organ transplantation: the risk of rejecting tissues from another person and the shortage of available organs. Any patient needing an organ transplant could benefit from regenerative medicine. Other potential patients include injured soldiers, accident victims, and those born with congenital defects [12].

However, ethical issues arise every time that a clinical trial is initiated, and these involve the rights of the patient, financial and nonfinancial conflict of interest, etc., and there will always be those who will raise ethical concerns on obscurantist or religious grounds. Certain aspects of regenerative medicine, however, make the established ethical concerns in clinical research particularly crucial in this new frontier. For example, the nature of regenerative research involves creating individualized therapies. In the typical development of a drug, researchers can apply knowledge gained from initial studies on a small group of people to the design of the next clinical study. Thus, the chances of harm to the participants in the next study decrease, while the

chances of potential therapeutic benefit increase. The individualized nature of regenerative medicine makes the conclusions drawn from one study less transferrable to the next study [12].

Whatever the case, this does not mean that there should be no research on regenerative medicine. This is the most exciting field in modern times as nature is slowly revealing that both the disease and the cure are hidden within the body. Care must be taken to ensure safety and benefit, and it is the brave persons who are venturing into unknown waters, who will discover the new vistas of medical science. Had Galileo been completely gagged, we may not have made the kind of advances in science that we have made today. Ethics is very important and so is moral judgment, but these should not stop progress toward a new horizon.

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